DEVELOPMENT AND EVALUATION OF AN ORAL CONTRACEPTIVE FOR WILD PIGS

A Thesis

by

SCOTT ROBERT CAMPBELL

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Duane Kraemer
Co-Chair of Committee,	Mark Westhusin
Committee Members,	Cheryl Dyer
	Gregory Johnson
Head of Department,	Larry Suva

December 2016

Major Subject: Biomedical Sciences

ABSTRACT

Wild pigs are an invasive species widely distributed throughout Australia and the Americas. Wild pigs are carriers of over 30 zoonotic diseases and are a major source of crop destruction, with damages associated with pigs estimated at $1.5 billion annually in the U.S. alone. Current mitigation techniques such as trapping and shooting are rendered ineffective due to the high fecundity of pigs. Recent population models suggest that decreasing fertility would result in sustainably reduced populations of wild pigs. No fertility control product currently exists for wild pigs, although the combination of triptolide and 4-vinyclohexene diepoxide (VCD) in ContraPest®, a rat contraceptive product, shows considerable promise. The objective of this project was to evaluate fertility control bait (FCB) containing triptolide and VCD for its potential use in pigs. To evaluate the effects of this FCB on boar fertility, males (n=5) were provided active FCB twice daily for 15 days. Semen parameters were monitored before (D0) and after (D37, D45, and D60). Males that freely consumed the FCB, had significant decreases ($p<0.05$) in viability, morphology, and progressive motility were observed at D37 and D45, indicating decreased fertility. By D60, parameters began to trend upwards and histological evaluation of the testes showed normal spermatogenic activity suggesting a transient effect on spermatogenesis. Females were provided placebo (n=6) or active (n=5) FCB twice daily for 15 days. Thirty days after cessation of the treatment period (D50), ovaries were collected from all females. A significant decrease ($p<0.05$) in both ovarian mass and prevalence of preovulatory follicles in treated gilts suggested a decline in ovulation rate. Histological studies found significant reductions ($p<0.05$) in all

immature follicular stages suggesting a prolonged decline in fertility. The results of these studies suggest the potential for developing an FCB, utilizing the combination of triptolide and VCD, to reduce the fecundity in both male and female wild pigs.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kraemer, my committee chair, and Dr. Westhusin, co-chair, as well as committee members Dr. Dyer and Dr. Johnson for their guidance and support throughout the course of this research. I am also incredibly thankful for SenesTech, Inc. for their funding and support to make this research possible. Further, I would like to thank Brandy Pyzyna for her additional mentorship throughout the course of this project.

I would also to thank all of my colleagues at the Reproductive Sciences Lab as well as the other graduate students within the College of Veterinary Medicine family. Finally, I would like to thank my parents and friends for their continued support throughout the entirety of this work.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 History of Wild Pig Populations

1.1.1 Rapid Expansion of Wild Pig Populations

Wild boars (*sus scrofa)* inhabit a wide range of habitats extending from Western Europe and Northern Africa to far eastern Russia and Southeast Asia. Domestication of wild boar occurred independently in the Middle East and Asia around 7,000 B.C. During the 1400s, European colonists brought domestic pigs to North America. Subsequently, through escapes and releases, these domestic pigs established free-roaming pig populations in North America. In turn, these free-roaming pigs became feral, or reverted to a state that closely resembles their wild ancestors. During the late twentieth century, game reserves in the United States imported wild boars from Europe. Shortly thereafter, a number of these individuals escaped and hybridized with the local feral pig populations. Currently in the United States, this hybridized wild pig population has reached an estimated size of 6.3 million individuals found across 41 states [1, 2].

During the colonization of Australia, European colonists also introduced domestic pigs [3]. Now feral pig populations are observed throughout Australia and neighboring islands such as New Zealand [4]. In 2008, the population of feral pigs in Australia had expanded to an estimated 24 million pigs [5]. Further, European colonization of South America led to similar domestic pig releases as seen in North America and Australia. In 2007, 91 municipalities in Brazil were documented to have feral pig populations [6]. By 2014, it was found that the number of municipalities in

Brazil with feral pig populations had increased to 472 municipalities [7]. Collectively wild boar and their descendants, feral pigs have established populations of wild pigs on all continents except Antarctica.

1.2 Significance of Wild Boar and Feral Pig Populations

1.2.1 Impacts on Agriculture and Public Health

The widespread occurrence of these wild pig populations has highlighted their numerous detrimental impacts. Farm fields are a frequent area of conflicts as they provide wild pigs easy access to a dense nutrient rich zone. Wild pigs' destructive foraging habitats can quickly trample large acreages of crops resulting in millions of dollars in damages. In the United States, damage caused by wild pigs on crops such as corn and peanuts results in an estimated $1.5 billion dollars in damages annually [8, 9]. Similar issues occur in Australia where wild pigs damage numerous crops including wheat and barley causing greater than $100 million in damages each year. It is common practice for some European countries to reimburse for crop damage attributed to wild pigs. For example, the Italian province of Siena pays on average $313,000 yearly to local agriculture operations due to wild pig crop destruction [10].

While direct damage to crops is a primary concern, secondary concerns include contamination of crops, as feral pigs are reservoirs of over 30 different zoonotic diseases. Wild pigs transmit *Salmonella enterica* and *Escherichia coli* (*E. coli*) through fecal material. Salmonella has been found in fifty percent of pigs in Texas and in wild pigs in 14 other states [11, 12]. Salmonella, a foodborne infection, is responsible for 20,000 hospitalizations and 400 deaths per year [13]. An outbreak of *E. coli* in 2007

resulted in 205 hospitalizations and 3 deaths. The source of the outbreak was determined to be spinach fields in Southern California that had been contaminated with wild pigs' fecal material [14].

Other diseases carried by wild pigs pose threats to the livestock industries. In areas of the United States, 44% of sounders or groups of reproductive sows (typically 2-6 individuals) and their young are carriers of pseudorabies [15]. Pseudorabies is asymptomatic in adult pigs but is lethal in piglets and an outbreak of pseudorabies would lead to vast economic damages to the pork industry. Currently, pseudorabies has been eradicated in United States commercial pig operations, however interactions between wild and domestic pigs have been observed, causing concerns that it could be reintroduced into commercial pig operations [16, 17].

1.2.2 Impacts on Environment

The destructive foraging habits of wild pigs not only effects the agriculture industry it also has a profound impact on the environment. These destructive foraging habits are the result of wild pigs using their snout to dig up or "root" up large chunks of dirt in search of small invertebrates such as worms and snails. Rooting has a detrimental impact on the growth of young plants and aids in the spread of invasive plant species [18, 19]. In California, high densities of wild pigs (2pigs/km^2) further reduce already low oak seedling regeneration rates [20, 21]. This is also commonly observed in areas of Poland as the presence of wild pigs reduced seedling regeneration rates 1.5 to 6 times normal [22]. Combined these foraging behaviors alter habitats results in diminished cover for small mammals such as voles, consequently reducing their populations [23].

Reduced populations of voles may disrupt ecosystem balance as they are a common prey item.

Wild pigs also directly affect populations of other larger vertebrates. In some areas of the United States and Australia, wild pigs will frequently dig up nests of endangered sea turtles [24, 25]. In Texas, nest predation also affects the population of bobwhite quail, where 28% of nests were destroyed by the local pig population [9]. Further, wild pigs will compete directly with other large games species such as deer potentially reducing their prevalence [26]. Ultimately, wild pigs pose a wide-ranging threat to the economy as well as a myriad of environmental issues.

1.3 Management Techniques

1.3.1 Nonlethal Management Techniques

The vast negative impact of wild pigs on the economy and ecosystems has led to the development of various techniques such as pig proof fencing and supplemental food sources, in an attempt to mitigate these damages. Traditionally, these pig proof fences consist of wire and mesh and are 28 inches tall. These fences are implemented to protect agricultural and sensitive environmental areas. In response to wild pigs damaging sea turtle nests, deployment of pig proof fences have been successful in protecting these critical habitats [27]. While effective, fence costs can be prohibitive when attempting to protect large areas of land. These fences typically cost $8,200-$21,325 per kilometer. Alternatively electronic fences are considerably cheaper ($2,000 per km) but they can be easily crossed by smaller pigs as electrical wires cannot be placed too closely to the ground as vegetative growth will cause disruptions in the current [27, 28]. Due to the

high cost of construction and upkeep of fencing, it is limited to application in small or high value areas.

Other strategies to control wild pig populations include providing supplemental food sources. These alternative food sources are placed in forested areas near farm fields in an attempt to discourage wild pigs from destroying crops. The effectiveness of this management technique has had mixed results. Several studies have indicated that supplemental feeding caused a decrease in agriculture damage [29-31] For example, in Italy as grapevines are nearing harvest, providing corn in adjacent forested areas resulted a 60% reduction in reported vineyard damages [32]. However, numerous other studies have been unable to produce similar results. More recent studies have found that supplemental feeding has proven to be insufficient in reducing the occurrence of crop damage [33]. Although damage may be mitigated immediately through supplemental feeding, the possibility lingers that in successive years damage may increase as a result of increased nutrient availability leading to improved survival and reproductive rates [34]. Independently fencing and supplemental feeding do not actively lower wild pig populations and are best coupled with strategies that actively reduce the prevalence of wild pigs.

1.3.2 Lethal Management Techniques

Actively removing pigs through lethal means such as shooting, trapping, or fencing are commonly utilized strategies to reduce wild pig populations. Wild pigs are popular game species throughout their native and introduced ranges. In areas with large wild pig issues, hunting is encouraged by local municipalities and many landowners hunt

wild pigs on their lands in an attempt to limit damages. However, recreational hunting efforts are largely ineffective in reducing wild pig populations regions [10, 35, 36]. This has led to the development of more organized ground hunting campaigns utilizing professional hunters. However, these were subsequently also ineffective at reducing local populations [37, 38].

In some areas, hunters have had success shooting from aircraft, allowing for large areas of land to be quickly covered. This has shown to be effective and can result in population reductions of 65% to 97% [38, 39]. Although effective, operating costs associated with aircraft are considerably high [40]. Recently, marketing aerial shooting as a recreational hunting method has been implemented in an effort to reduce cost. Additionally, in areas that provide wild pigs with large amounts of cover, in national parks, or in urban areas aerial shooting is not feasible and require less intrusive management techniques.

Trapping is a considerably less intrusive method to lower wild pig populations. The most commonly used trap style is a corral style trap that consists of a large circular pen typically baited with corn. The entrance of these traps contains a sliding door that closes by placing a pressure trigger inside the pen or via camera monitoring. Landowners independently implement traps, which limits effectiveness due to sporadic application over large areas. Field studies evaluating the effectiveness of trapping campaigns found 90-100% drops in consumption rates of bait in traps. A subsequent investigation found only 80% reductions in populations, indicating a portion of the population refused to enter traps (trap shyness) [38, 41]. Other studies observed up to

48% of animals would not enter traps [42]. The social structure of pigs is a major influence on trapping efforts as sounders are more efficiently trapped in comparison to boars that are solitary by nature [43]. The large proportions of animals exhibiting trap shyness and the social structure of wild pigs limits the effectiveness of trapping.

The ineffectiveness of shooting and trapping campaigns led to the development of alternate strategies such as poisoning. For poisoning to be effectively used to reduce wild pig populations, O'brien [44] described seven traits needed for an effective pig poison. These traits are that the poison must have a high toxicity, consumed readily, commercially available, does not persist in the environment, has a low hazard to distributors, low cost, and have an acceptable time of death.

In Australia, PIGOUT® is the most extensively employed poison, but is not approved for use in the United States. The active ingredient in PIGOUT®, sodium flouroacetate, is quickly metabolized to flourocitrate after consumption [45]. Flourocitrate acts by inhibiting the enzyme aconitase of the Krebs cycle [45]. PIGOUT® triggers emesis in wild pigs thereby decreasing the ingested dose of PIGOUT® while subsequently posing a threat to non-target species who consume the vomitus. In an attempt to limit emesis, PIGOUT® utilizes a cylinder design that has an outer layer of a cereal and meat attractant and an inner layer of a proprietary matrix containing sodium flouroacetate. The inner layer was designed to slow the release of sodium flouroacetate in the stomach but did not prove effective in reducing vomiting [46].

Recently, the public perception of PIGOUT® poisoning has come into question as PIGOUT® induces side effects that are considered inhumane such as convulsions [47].

This negative public perception of PIGOUT® led to the development of a new toxin, sodium nitrite. Sodium nitrite has considerable promise to control wild pig populations, as it is considered to induce a humane death. Sodium nitrite acts by increasing the prevalence of methemoglobin, a type of hemoglobin which cannot transport oxygen and typically makes up 1% of total hemoglobin [48]. After consumption of sodium nitrite baits, wild pigs will quickly become unconscious, and death follows shortly after when levels of methemoglobin increase over 70%, which inhibits the body's ability to transport oxygen [49].

While sodium nitrite has potential to be a humane poison, it is a bitter compound and wild pigs will not consume lethal doses. Much of the research involving the use of sodium nitrite as a poison focuses on creating a bait that contains a lethal dose. Currently, various formulations using microencapsulation to disguise the bitter taste during consumption are being evaluated [50-53]. In New Zealand, researchers have developed a peanut flavored paste bait that stimulates consumption of lethal doses. [50]. In 2015, sodium nitrite was approved for use by the New Zealand government with further studies being completed for registration in the United States and Australia [52, 53].

The crux of the problem with poisoning is that these rapid reductions in pig populations from poisoning campaigns are not sustainable or cost effective. Previous studies in Australia subjected a wild pig population to PIGOUT® poisoning, the population was reduced by 58% but a year later the population had already surpassed the initial population size. For a poisoning campaign to be successful, population models

suggest a population reduction of 95% would need to occur, and followed by an intensive hunting program to remove survivors [54]. These models have found that even a 70% decrease in population would completely recover in 2.5 years [54].

1.4 Wild Pig Reproductive Physiology

1.4.1 Wild Pig Reproductive Biology

The ability to rebound after large population reductions is due to the efficient reproductive systems of wild pigs. Sows produce large litters of 4-6 pigs with a gestation period of 115 days. Wild pigs that have a higher prevalence of domestic pig genetics have slightly larger litter sizes as artificial selection during domestication resulted in increased reproductive capabilities [55]. Following a large removal event, an overabundance of nutrients allows for an increase in body condition in surviving animals, which correlates with improved reproductive efficiency[56, 57]. As a result of these optimal conditions, sows are capable of producing up to three litters in 14-16 months [54].

Wild pigs in native European ranges typically breed seasonally, with mating occurring in late autumn and farrowing or parturition occurring in spring. Wild pig populations in the southern United States have two peak farrowing times during February and July, but farrowing is observed throughout the year [58]. In Australia, wild pigs also breed throughout the year but peak in summer months [59]. This difference in breeding behavior from European populations to feral populations is due to a combination of increased prevalence of domestic pig genetics and temperate habitat.

Piglets rapidly mature, male offspring become fertile between 5 and 7 months of age while female sexual maturation is considerably more variable [60, 61]. Due to intraspecific competition between boars, younger boars are frequently outcompeted by older larger boars and do not breed until around 12-18 months of age [62]. Genetic studies have observed that older larger boars are more likely to successfully breed in comparison to smaller counterparts [63].

Sows typically become fertile between 5 and 12 months of age, which is thought to be dependent on body weight and it has been suggested that sows must reach a size of 20-35kg to be fertile [64, 65]. Once sexually mature, sows will come into estrous every 21 days and will be receptive to mating attempts from boars for two to three days.

1.4.2 Potential for Fertility Control

Disrupting the high fecundity of wild pigs may be the key to sustainable reductions in populations. Biological models simulating wild population dynamics, found the variable with the most impact on population size was the number of litters and piglets produced per year [66]. Further models demonstrated that rendering fifty percent of females infertile would cause a fifty percent reduction in the population in two years and an 80% reduction in five years [67]. Models evaluating the potential of fertility control found that the application of a fertility control agent in sows could halt population growth and when coupled with removal of animals through hunting would cause a sustainable reduction in populations [68]. Collectively, these models indicate that decreasing fertility of wild pigs is a promising avenue that requires further investigation.

Fertility is largely dependent on the production of viable gametes through gametogenesis; inhibition of this process would greatly reduce fertility. Gametogenesis occurs through unique gender-specific mechanisms. In the male, this is referred to as spermatogenesis and in the female as folliculogenesis. Little literature exists elucidating the differences in gametogenesis between wild and domestic pigs but the few studies that have investigated this have found little difference in comparison to domestic counterparts [69]. As previously mentioned, varying levels of domestic pig genetics occur in wild populations which creates considerable overlap. For the purposes of this review, gametogenesis and its regulation in domestic pigs will be reviewed.

1.5 Gametogenesis and Endocrine Regulation

1.5.1 Spermatogenesis

At the onset sexual maturity boars, begin producing a constant supply of spermatozoa. Spermatogenesis consists of three phases: proliferation, meiotic, and spermiogenesis phases, respectively. Within the testes, spermatogenesis begins in the seminiferous tubules. Spermatogenesis initiates within the basement membrane of the seminiferous tubules. It is here that proliferation of mitotically dividing spermatogonial stem cells occurs. Spermatogonia undergo typically six divisions prior to entering meiosis [70]. At the onset of meiosis, spermatogonia are referred to as spermatocytes. Once the initial cellular division of meiosis occurs, primary spermatocytes transition to secondary spermatocytes and enter the second division of meiosis resulting in a round haploid cell known as a spermatid.

Spermatids enter the final phase of spermatogenesis and undergo a complex remodeling that enables the spermatid capable of surviving the female reproductive tract to complete fertilization, referred to as spermiogenesis. During transformation, the nucleus exchanges histones, proteins used for packaging DNA in all cells, for protamines. This exchange allows for tight compaction and inactivation. Partly surrounding this compacting nucleus is the developing acrosome. The acrosome is a necessary component of the head region that covers a majority of the anterior region of the compacting nucleus. The main function of the acrosome is to break down the zona pellucida that surrounds the ovum. Coinciding with the developing acrosome, flagellogenesis begins. During flagellogenesis, mitochondria migrate to the posterior end of the nucleus, and centrosomes elongate to form the flagella. At the base of the flagella, mitochondria condense and form the mitochondrial sheath.

The spermatid, now referred to as a spermatozoon, contains a head region made up of a condensed nucleus surrounded by the acrosome on the anterior portion. The posterior region of the nucleus contains the basal plate, which anchors the flagella to the nucleus. Distally to the basal plate, the mitochondrial sheath condenses around the flagella that will provide the necessary energy for motility. Beyond the midpiece, is the principle piece that contains the remaining portion of the flagella. Once spermatogenesis is completed, the sertoli cell releases the spermatid into the lumen of the seminiferous tubules.

Upon completion of spermiogenesis, immature spermatozoa are not motile. Peristaltic contractions move these immature spermatozoa into the epididymis, which

provides the necessary conditions required for the final maturation steps of the spermatozoa. Lining the tubules of the epididymis are various cell types that are required to support further maturation of the spermatozoa. Clear and narrow cells maintain the necessary acidic pH of the epididymal lumen required for maturation while basal cells protect spermatozoa from oxidative stress [71]. Principal cells contribute various secretory products such as ions and proteins to the lumen [71]. The completion of maturation takes approximately 10 days in pigs but can occur in as little as 2-5 days [72]. From the initial division of spermatogonial stem cells to ejaculation of mature spermatozoa occurs in ~50 days.

1.5.2 Folliculogenesis

Unlike the boar that has spermatogonia constantly proliferating to provide a continuous supply of spermatozoa, the analogous proliferation event in the sow occurs in a short window during fetal development. Primordial germ cells undergo multiple mitotic divisions, peaking at 50 days into development [73]. As mitosis ceases, the developing ovary is left with a finite supply of primordial germ cells. At this point primordial germ cells enter meiosis and begin to transition into oocytes. Meiosis arrests in pigs in the diplotene stage. During this process, fifty percent of primordial germ cells are lost to atresia [73]. This retention rate in domestic pigs may be due to artificial selection and could be lower in wild pigs.

By 70 to 90 days into fetal development, one or two layers of granulosa cells surround the immature oocytes to form the primordial follicular structure [74]. In these structures, immature oocytes will remain quiescent until recruitment. A currently not

well understood stimulation on the surrounding granulosa cells activates the mechanistic target of the rapamycin complex 1 (mTORC1) and KIT ligand (KITL) [75, 76]. mTORC1 triggers differentiation of the surrounding granulosa cells while KITL binds to KIT membrane receptors to trigger oocyte growth [75, 76].

This stimulates the development of the primordial follicle into a primary follicle, characterized by proliferation of one to three layers of granulosa cells. Additionally, the zona pellucida begins to develop around the maturing oocyte. Granulosa cells continue to proliferate and once 3-20 layers of granulosa cells have been accumulated, it reaches the secondary follicular stage and is 0.14-0.4mm in diameter [77]. At this point, the granulosa cells must develop FSH receptors to for continued growth. Approximately 50 follicles are recruited and continue on to the antral stage of follicular develop, developing a theca cell layer surround the granulosa cells [78]. Additionally, a fluid filled antral space begins to form around the oocyte that contributes substantial volume resulting in a follicular diameters of 1-6mm [78]. From this recruited cohort, 12-20 follicles are selected for continued growth and ovulation and reach a diameter of 8-10mm. For selection to occur, a follicle's theca cells must synthesize LH receptors [79]. After ovulation, the remaining granulosa cells undergo luteinisation and transition into a corpus luteum (CL).

1.5.3 Endocrine Control of Gametogenesis

Gametogenesis in both genders is dependent on cyclic releases of hormones from endocrine glands for proper function. The hypothalamus releases pluses of gonadotropin releasing hormone (GnRH) which travels through the hypophyseal portal system and

stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In the male, LH stimulates testosterone production by Leydig cells. These specialized cells are located in the interstitial space surrounding the seminiferous tubules of the testes. Testosterone is required for spermatogenesis and is concentrated within the seminiferous tubules by sertoli cells producing androgen binding protein. Sertoli cells also produce inhibin and are stimulated by FSH to convert testosterone to estradiol. Estradiol as well as testosterone negatively feedback on the hypothalamus to inhibit the release of GnRH. Over a course of several hours, the levels of estradiol and testosterone decrease, lifting the inhibition of GnRH release and beginning another cycle approximately every 4 hours.

The corpora lutea dictates the sow's estrous cycle and fate of developing follicles. This period of progesterone secretion by the corpus lutea is known as the luteal phase and consists of the initial 13-15 days of the sow's estrous cycle. During this time the sustained secretions of progesterone negatively feedback to the hypothalamus and anterior pituitary to inhibit the release of GnRH, FSH, and LH. Throughout the luteal phase, activation of primordial follicles continues in a wavelike manner. Follicles that reach the late secondary stage during the luteal phase will undergo atresia due to suppression of FSH [78]. Between 12 and 14 days after ovulation, the corpora lutea develop prostaglandin receptors [80]. If pregnancy does occur, the developing conceptus produces estrogens that direct the release of prostaglandin into the lumen of the uterus [81]. If pregnancy does not occur, approximately around 15 days after ovulation the endometrium of the uterus will release prostaglandin into the circulatory system that triggers luteolysis of the corpora lutea [81].

The destruction of the corpora lutea ceases progesterone secretion and removes the block on the hypothalamus and pituitary and the sow transitions into the follicular phase of the estrous cycle. During the follicular phase, FSH supports the growth of late secondary follicles [78]. The theca cells of these large follicles produces androstenedione that diffuses into the granulosa cell layer which converts it to estradiol. The levels of estradiol continue to increase before peaking at day 19 that stimulates a surge of LH needed for ovulation at day 21 of the estrous cycle. After ovulation, the formation of new corpora lutea restarts the estrous cycle.

1.6 Fertility Control in Vertebrate Pests

1.6.1 Considerations for Fertility Control

Currently fertility control is receiving increased attention as a potential solution to manage mammalian pest populations. For effective fertility control implementation, it will have no adverse side effects, will inhibit fertility for at least one reproductive season, and ideally work in both sexes. Further, fertility control strategies should be cost effective in production and distribution, have no adverse impacts on ecosystems, limit consumption by non-target species, and be stable under a wide range of field conditions. [82, 83]. At this time, there is not a fertility control method approved for use in wild pigs. The conserved nature of mammalian reproduction allows fertility control strategies to work in multiple species.

1.6.2 Immunocontraceptives

One such method to decrease fertility is through injectable immunocontraceptives, which utilize the body's own immune system to create

antibodies that target specific reproductive proteins and hormones. For example, fertilization requires the spermatozoa to bind to and penetrate the zona pellucida (ZP) that surrounds the ovum. The porcine ZP (PZP) immunocontraceptive stimulates the body to produce antibodies that bind to the zona pellucida of the ova preventing spermatozoa adhesion thereby blocking fertilization [84]. This has been shown to be effective in numerous mammalian species but not in wild pig

Although PZP was ineffective in wild pigs, Gonacon^{TM}, another immunocontraceptive strategy has proven effective in captive wild pigs. Both spermatogenesis and folliculogenesis rely upon the stimulatory action of GnRH. Gonacon^{TM} acts by stimulating the immune system to produce GnRH antibodies thereby blocking the release of FSH and LH from the pituitary [85]. Wild sows receiving this vaccination were infertile for up to six years [86]. No studies have evaluated the impact of GonaCon^{TM} on wild boar fertility but early trials in domestic pigs resulted in reductions of testes volume and plasma testosterone. Histological evidence in the testes also suggested a negative impact on spermatogenesis [87]. However, while Gonacon^{TM} was effective in reducing fertility of wild pigs, injectable immunocontraceptives are not suitable for field application. Administration of injectable vaccines would require an extensive trapping program that would have considerable labor costs. Adaptation of Gonacon^{TM} into a similar product as RABORAL V-RG, a system designed to vaccinate foxes, coyotes, and raccoons through coating of the mucosa membrane of the oral cavity could prove more efficacious for field use [88].

1.6.3 Fertility Control Bait

It would be advantageous for a bait that upon consumption reduces the reproductive capabilities of wild pigs. Distribution of a fertility control bait (FCB) over large areas would be less invasive then large-scale hunting and an efficacious method to administer to a large proportion of the population in comparison to trapping and vaccination. Early attempts by Sanders *et al* [89] to create a FCB in wild pigs using the industrial chemical ERL-4221, a potentially ovotoxic compound, were unsuccessful as sows showed no reduction in ovarian follicles. It is possible that a higher dose of ERL-4221 is necessary to induce reductions in ovarian follicles, but the design of the bait and the bitterness of ERL-4221did not allow for investigating higher doses. Increasing the concentration of ERL-4221 in the bait led to adverse reactions during consumption including rolling in the bait and excessive salivation.

Since this time, a fertility control product for rats has been developed and is showing considerable promise as it has been recently approved for use by the Environmental Protection Agency (EPA) [90]. This product contains two active ingredients, triptolide and 4-vinylcylcohexene diepoxide (VCD). Triptolide, a diterpenoid diepoxide, is isolated from the ancient Chinese medicinal plant *Tripterygium wilfordii* (figure 1.1). Historically, triptolide has been utilized clinically to treat rheumatoid arthritis and systemic lupus due to its anti-inflammatory and immunosuppressive properties [91, 92]. Recently, triptolide has been receiving increased attention as a potential treatment for cancer [93]. The other active ingredient, VCD is an

industrial chemical that has been previously used to create a menopausal rodent model [94, 95].

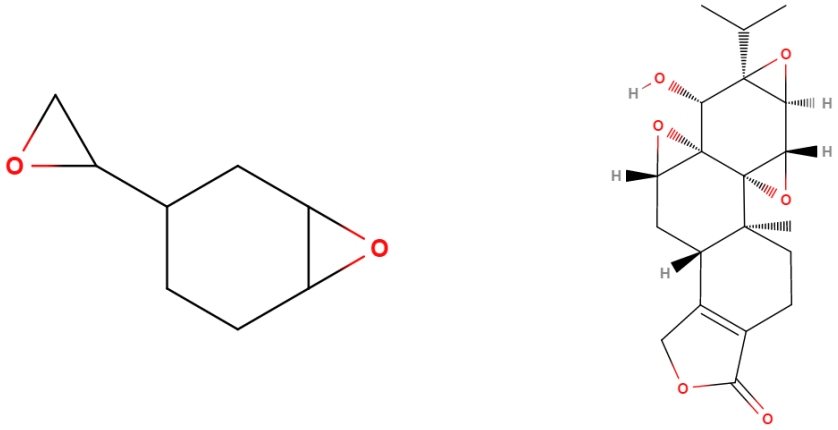

Figure 1.1 Triptolide and VCD. Left, chemical structure of 4-vinylcyclohexene diepoxide (VCD). Right, chemical structure of triptolide

Both triptolide and VCD impair gametogenesis in both sexes. In the female, the immature oocyte within the primordial follicle is maintained by the surrounding granulosa cells through kit-ligand (KITL) and c-kit paracrine signally. KITL functions to prevent apoptosis and trigger follicle activation [96]. VCD disrupts this process by

reducing c-kit mRNA expression on the plasma membrane of the immature oocyte thereby decreasing KITL stimulus. Intraperitoneal injection of VCD in rats (160mg/kg) for fifteen days, resulted in depletion primordial follicles after 15 days, and depletion of primary follicles occurred 31 days after treatment [94]. The breakdown in communication between the granulosa cells and oocyte causes follicles to undergo atresia. As a result, a decrease in the prevalence of primary follicles occurs, exasperating the effects of VCD as primary follicles produce anti-müllerian hormone (AMH), which acts to inhibit primordial follicle activation. Reductions in the inhibitory action of AMH allows more primordial follicles to attempt to activate but are inhibited by the decreased KITL stimulation [97].

During this time, follicles that mange to escape VCD-induced atresia and reach the secondary stages become targets of triptolide as it induces apoptosis of the granulosa cells that are supporting the oocyte during folliculogenesis [98]. Triptolide is detoxified by cytochrome P450s (CYPs), this process generates reactive oxygen species (ROS) that if not properly metabolized can damage plasma membranes, DNA, and cellular metabolism [99, 100]. Cellular processes have several defense mechanisms to remove ROS, but repeated dosing of triptolide overwhelms them and leads to the accumulation of ROS that leads to oxidative stress induced apoptosis. This was confirmed by Zeng *et al.* [101] as apoptotic granulosa cell's endoplasmic reticulum were producing 78-kDa glucose-regulated protein (GRP78) indicative of oxidative stress induced apoptosis [101, 102]. Together, VCD and triptolide form a two-prong approach to accelerate the depletion of ovarian follicular reserves and inhibit the growth of large follicles

continuing on to ovulation. Consumption of the proprietary FCB containing both triptolide and VCD developed by Dyer *et al* [90] resulted in declines of all ovarian follicular stages and no corpora lutea were observed.

Currently, there is no literature observing the dual effects of triptolide and VCD on male fertility. However, Huynh *et al* [103] observed dosing male rats for 70 consecutive days with triptolide rendered male rats sterile. At this point, epididymal sperm concentration had reduced 85% and all spermatozoa were immobile [103]. Additionally these immobile spermatozoa had severe morphological defects such as head-tail separation [104]. Interestingly, 14 weeks after cessation of treatment, four of six male rats' fertility recovered. Upon closer examination after the 70-day treatment period, reduced spermatogenic activity was observed within the seminiferous tubules but spermatogonial stem cells persisted. These results suggest a transient effect on spermatogenesis that spares spermatogonial stem cells, which would explain the observed recovery after cessation of treatment. Additional studies since this time have shown triptolide to have similar reductions in epididymal spermatozoa concentration and motility after only seven days of treatment [105]. This indicates that triptolide also acts to disrupt epididymal sperm maturation.

Triptolide acts to reduce spermatogenic activity, but how it acts in the epididymis and testes remains unclear. Previous *in vitro* studies have observed that Leydig cells are susceptible to triptolide induced apoptosis, which would suggest decreases in testosterone that would explain decreases in spermatogenesis [106]. Ma *et al* [107]

demonstrated that triptolide treatment alters metabolic pathways of the supporting sertoli cells.

A previous study examining the effects of VCD administration in male rats found disruptions in early stages of spermatogenesis by inhibiting the proliferation of spermatogonia and meiotic divisions of spermatocytes, manifesting in significant reductions in concentrations of epididymal spermatozoa [108]. The effect appears to be transient as spermatogonial stem cells persist and recovery occurs after 60 days post treatment [108]. At this time, the mechanism of VCD inhibiting spermatogenesis is unknown. Overall, triptolide and VCD have been shown to disrupt early and late stages of gametogenesis in both sexes of several mammalian species but their effects in pigs have yet to be evaluated [90, 103, 109, 110].

1.7 Development and Evaluation of a FCB for Wild Pigs

1.7.1 Development of a FCB

For this FCB to be effective in pigs, consumption must occur in large enough amounts so high enough doses of triptolide and VCD are ingested. Previous field studies have observed that fish and peanut butter flavors are common bait additives [51, 111, 112]. Additional studies observed pigs preferred strawberry and other fruit flavored baits [113]. Jointly, this suggests that a palatable bait should include fat-rich and sweet flavors. This lead to the development of a proprietary FCB that is a liquid that largely consists of sweetener and vegetable oil as wild pigs prefer sweet (artificial fruit) and fat-rich (peanut butter and fish) flavors [111, 113].

To evaluate the palatability and efficacy of the FCB, this study was composed of four individual experiments. The first experiment, the preference trial, focused on developing and evaluating a palatable FCB. Initially, the preference trial utilized inactive bait, without the active ingredients triptolide and VCD, to determine if pigs would prefer liquid or food-based bait. This was examined as it is possible that a liquid bait will be efficacious in regions where water is a limited resource.

Based upon the results of the preference trial, the bait that is most palatable will be used during the second experiment. If after the preference trial, no bait is determined to be palatable, modification of the bait will occur. The second experiment was designed to evaluate the palatability of the active ingredients, triptolide and VCD. If the active bait was determined to be palatable, it would be utilized in the third and efficacy experiments to evaluate impacts on pig fertility.

1.7.2 Efficacy of FCB

The third experiment was designed to evaluate long-term consumption of active FCB and reductions in fertility in boars as a result. Evaluating artificially collected ejaculates before, during, and after the feeding period will allow for monitoring of changes in seminal parameters indicative of fertility. Additionally, observing testes volume will allow for evaluation of potential changes in spermatogenesis that manifest by altering the size of the testes. Further, the endocrine system will be monitored through monitoring of plasma testosterone levels (figure 1.1). Due to triptolide and VCD's dual action in the testes and epididymis, we hypothesized to find a decrease in

seminal quality, testes volume, and testosterone levels suggestive of a reduction in fertility.

Figure 1.2 Boar efficacy timeline. Experimental Design of the boar efficacy study. Top arrows indicate how long each phase. Below, Day (D) indicates at what point data fertility parameters were collected.

The fourth experiment will evaluate long-term consumption of the active FCB and changes in gilt fertility as a result. Fertility will be assessed through evaluation of parameters indicative of potential fertility in comparison to gilts receiving inactive bait. Before, during, and after the 15-day active feeding period, serum samples of AMH were analyzed as an indicator of changes in primordial follicle reserve (figure 1.2). Further, the density of follicles in ovarian tissue of control and treated animals was determined under histological evaluation. The mass of an ovary is an indicator of the relative presence of large antral follicles and was compared between the two treatment groups at the completion of the study. Additionally, the amount and diameters of observable

follicles on the surface were measured to estimate antral follicle populations. We hypothesized to find a decrease in the amount of observable follicles and mass of the ovary as well as decreases in follicle densities and serum AMH levels indicative of follicle depletion.

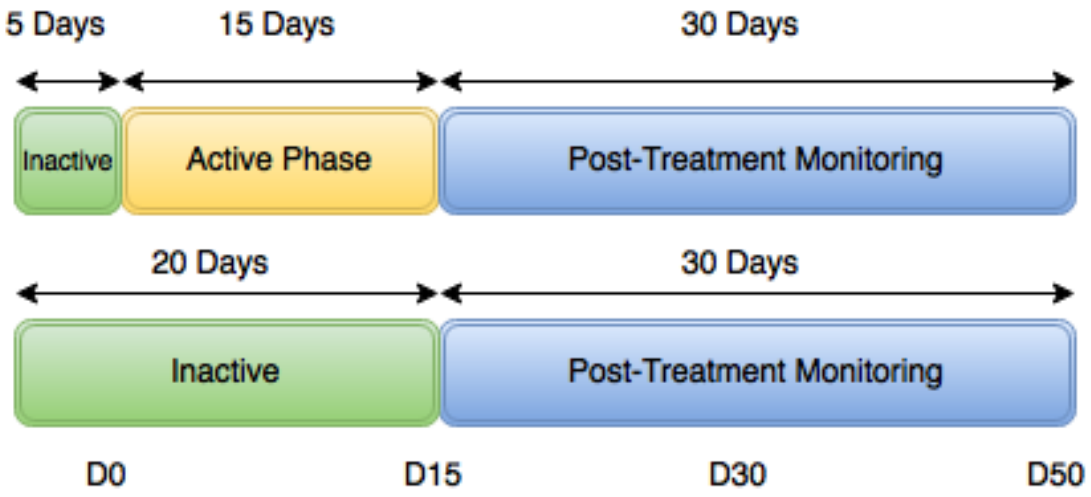

Figure 1.3 Sow efficacy timeline. Experimental Design of the sow efficacy study. Top of figure outlines the length of each phase in treatment animals. Bottom of figure outlines length of each phase in control animals. Day (D) indicate at what point blood was drawn for AMH assay

CHAPTER II

MATERIALS AND METHODS

2.1 Preference Trial

2.1.1 Animal Care

This study was completed at the Texas A&M Veterinary Medical Park (VMP) in College Station, Texas. Six Sinclair pigs (three boars and three sows) between one and four years of age were sourced from Texas A&M University Reproductive Sciences Laboratory colony housed at VMP. The animals were housed individually inside a temperature-controlled environment to minimize weather-induced variables. All animal handling, housing, and care procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M (2015-0155).

2.1.2 Bait Manufacturing

The proprietary inactive liquid FCB consisted largely of water, vegetable oil, surfactant, and sweetener. Baits were manufactured by Senestech, Inc.'s production facility located in Flagstaff, Arizona.

2.1.3 Feedings

Each pig was provided each treatment for five days with two days rest between treatments. The rotation through each treatment was randomized. Water was withheld overnight (~12 hours) prior to treatment. The four treatments consisted of inactive bait, water, water plus pig feed, and control bait plus pig feed. The amount of liquid (water or inactive bait) was determined through a ratio of 0.5mL of liquid to one gram of daily

ration of pig feed. For treatments that included feed, half of the daily ration of pig feed was mixed with the inactive FCB.

2.1.4 Observation Scores

All feedings were viewed by three observers for 30 minutes or until the entire treatment was consumed (which ever occurred first). In each case, the feeding behavior was scored and described, including an estimate of the amount consumed. Palatability scores were assigned to each as 1, 2, 3 or 4. Score 1 = consumed readily, high palatability; 2 = consumed some, medium palatability; 3 = consumed reluctantly, low palatability; 4 = consumed very little, non-palatable.

2.2 Palatability of Active Ingredients

2.2.1 Animal Care

This study was completed at VMP in College Station, Texas. The same six Sinclair pigs (three boars and three sows) used in the initial palatability study were use in this study. These pigs were sourced from Texas A&M University Reproductive Science Laboratory colony housed at VMP. The animals were housed individually inside a temperature-controlled environment to minimize weather-induced variables. All animal handling, housing, and care procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M (2015-0351).

2.2.2 Bait Manufacturing

Inactive and active liquid FCB was modified from the original formulation to include gelatin and strawberry flavoring. Gelatin was added to solidify the bait into 250g blocks based on the results from the initial palatability study. Strawberry flavoring was

added as it was highly palatable in previous bait studies with wild pigs [113]. Active FCB (250g) contained the two active ingredients triptolide (0.25mg) and VCD (54.25mg) respectively. Baits were obtained from Senestech, Inc.'s production facility located in Flagstaff, Arizona.

2.2.3 Feedings

The objective of this experiment was to determine if the inclusion of the active ingredients in FCB would be less palatable in comparison to inactive bait. Pigs were alternated between inactive and active FCB (figure 3.1) to determine if a negative reaction occurred during active consumption leading to avoidance of bait independent of treatment type.

Day 1 Inactive	Day 2 Inactive	Day 3 Active	Day 4 Active	Day 5 Active	Day 6 Inactive	Day 7 Inactive
Day 8 Active	Day 9 Active	Day 10 Active	Day 11 Inactive	Day 12 Active	Day 13 Active	Day 14 Active

Figure 2.1: Feeding schedule of the secondary palatability study.

Feedings occurred twice daily, 8 hours apart. Preliminary feeding studies found pigs preferred the solidified FCB cut into small pieces. Inactive and active FCB (250g blocks) were cut into 2cm cubes and mixed with half of each pig's daily ration of feed during each feeding period. Feedings were observed for 30 minutes to identify

differences in feeding behavior and to monitor health. Any remaining bait after the 30-minute feeding period was removed and weighed to estimate total consumption.

2.3 Boar Efficacy Study

2.3.1 Animal Care

The study was completed at VMP in College Station, Texas. Four Sinclair boars around one year of age were sourced from Texas A&M University Reproductive Science Laboratory colony housed at VMP. Due to limited availability, an additional three commercial boars (8 months of age) were purchased from the Texas Department of Corrections (TDOC). Boars were individually housed inside a temperature-controlled environment to minimize weather-induced variables on consumption and fertility parameters. All animal handling, housing, and care procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M (2015-0351).

2.3.2 Fertility Control Bait

Inactive and active FCB was obtained from Senestech, Inc. Active FCB (250g) contained triptolide (0.25mg) and VCD (54.25mg).

2.3.3 Feedings

Seven boars (four Sinclair and three commercial) were initially provided inactive FCB twice daily for five days (inactive phase) to adjust to FCB. Immediately following, boars were provided active FCB for 15 consecutive days (active phase) (figure 1.1). Feedings consisted of 250g of 2cm cubes of inactive or active FCB. Feedings were observed for thirty minutes or until all bait was consumed.

2.3.4 Semen Collection

Boars were trained to mount a "dummy" and semen was collected using the gloved hand technique at day 0 (day prior to receiving active), 7, 15, 30, 37, 45, and 60. The sperm rich fraction of the ejaculate (appears milky) was collected into a thermoinsulated cup lined with a semen collection bag to maintain temperature during collection. Positioned across the opening of the thermoinsulated cup was sterile gauze to filter out seminal plug. After collection, the semen collection bag was immediately placed in a 35° water bath and transported to the laboratory for immediate analysis.

2.3.5 Sperm Viability Stain

To evaluate changes in viability, 5µl of semen was placed onto a pre-warmed slide that contained a mixture of 5µl of 10% Nigrosin (blue) and 5µl of 5% Eosin Y (red). The stained sperm sample was smeared across the slide using a second slide and was dried using a hair dryer. Fifty spermatozoa were observed under 100x (oil immersion) by counting 10 spermatozoa per view. Spermatozoa were classified as viable (if neck region did not take up stain) or non-viable (spermatozoa did take up the stain, appeared pink).

2.3.6 Sperm Morphology and Abnormalities

Sperm abnormalities were observed using the previously prepared Nigrosin and Eosin stain. Spermatozoa were categorized as either normal or into three groups depending on the location of the abnormality as either head, neck, or tail abnormalities. Fifty spermatozoa were counted by the same method used in the sperm viability stain.

2.3.7 Progressive Motile Spermatozoa (PMS)

To observe progressive motility, fresh semen sample (5µl) was placed onto a pre-warmed microscope slide and cover slipped and observed under 40x magnification at the same time points as viability and morphology. Five views were observed and the percentage of spermatozoa with progressive motility was estimated and placed onto a scale 0-5; 0= 0-10%, 1= 10-25%, 2= 25-50%, 3= 50-70%, 4= 70-90%, and 5=90-100% as previously outlined by Martinez *et al.* [114].

2.3.8 Semen Concentration

Five µl of the semen sample was diluted into 995 µl of water (1:200 dilution). Ten µl of the dilution was placed into each well of a hemocytometer. The hemocytometer was viewed under 40x magnification and the five diagonal blocks were counted. The average between the two counts was averaged and concentration was calculated using the following formula:

- Concentration (Sperm/mL of semen) = N x 5 x DF x 10,000

N = the averaged count from the hemocytometer. Five (5) = correction factor from counting only 5 of the 25 squares. DF = dilution factor, which was 200 in this experiment. 10,000 = correction factor due to the volume beneath the cover slip of 0.0001 ml per chamber.

2.3.9 Testes Volume

Testes volume measurements of each boar was taken at day 0, 15, 30, 45, and 60. Testes volume was calculated by measuring the length and width of each testes. Measurements from the right and left testes were averaged. Volume was estimated using

the formula for a prolate sphere, $V=4/3\pi ab^2$. Where V= Volume, a= a axis or half of the length, b= b axis or half of the width (Figure). Average volume between individual testes was divided by bodyweight to calculate gonadosomatic index (GSI).

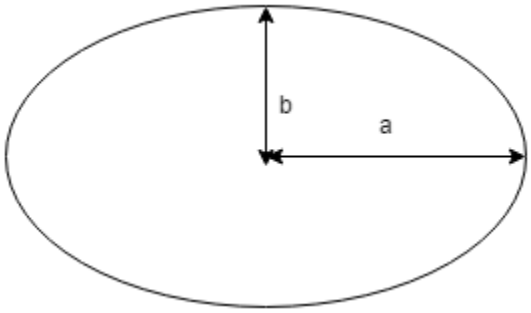

Figure 2.2 Prolate sphere. The volume of a testes, a prolate sphere is calculated by $V=4/3\pi ab^2$. Where "b" is half of the width and "a" is half of the length

2.3.10 Testosterone Levels

Blood (10ml) was collected from either the cranial vena cava or the brachiocephalic vein at Day 0, 15, 30, 45, and 60. Blood was centrifuged at 2000 rpms for 45 minutes. Serum was separated from the hematocrit and divided into 1mL aliquots and frozen at -80°C until testing. Testosterone assay was performed by the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL).

3.3.11 Testes Histology

Testes biopsies were collected and placed into modified Davidson's solution for 48 hours. Fixed tissue was then placed in 70% ethanol until processing. Processed tissue was embedded in paraffin was and sectioned at 4μm. Representative samples from each individual was placed on a slide and stained with hematoxylin and eosin.

2.4 Sow Efficacy Study

2.4.1 Animal Care

This study was completed at VMP in College Station, Texas. Three cyclic Sinclair gilts (6-12 months of age) were sourced from Texas A&M University Reproductive Science Laboratory colony housed at VMP. Due to limited availability, nine additional cyclic commercial gilts (6-12 months of age) were purchased from TDOC. Gilts were individually housed inside a temperature-controlled environment to minimize weather-induced variables on consumption and fertility parameters. All animal handling, housing, and care procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M (2015-0351).

2.4.2 Fertility Control Bait

The same solidified strawberry flavored bait as used during the secondary palatability study was used in this study. In the active FCB, concentrations of triptolide and VCD were 0.25mg of triptolide and 54.25mg of VCD, respectively. Baits were manufactured by Senestech, Inc.'s production facility located in Flagstaff, Arizona.

2.4.3 Feedings

Twelve gilts (three Sinclair and nine commercial) were initially provided inactive FCB twice daily for five days (inactive phase) to adjust to FCB. Next, gilts were divided evenly into inactive (four commercial and two Sinclair) and active (one Sinclair and five commercials) groups (Figure 1.2). Feedings occurred twice daily, approximately eight hours apart. Feedings consisted of 250g of 2cm cubes of inactive or active FCB. Bait was mixed in with half of each boar's ration of dry feed. Feedings were observed for

thirty minutes or until all bait was consumed. Remaining bait after the thirty minute feeding was removed and weighed.

2.4.4 Ovary Collection and Large Follicle Measurements

Ovaries were removed and infundibulum, fat, or non-ovarian tissue was trimmed. Left and right ovaries were weighed and diameters of all large growing follicles were measured and classified. Visible surface follicles were measured using calipers and classified as small (<3.00mm), medium (3.00-6.900mm), or large (>7.00mm) [78].

2.4.5 Ovarian Histology and Follicle Counts

After removal, weighing, and surface follicular measurements, ovaries were cut in half longitudinally with a scalpel and placed into a solution of 10% neutral buffered formalin. Halves of each ovary were removed, crosswise to create approximately 5mm thick sections and placed into individual cassettes. Ovarian tissue was processed in ascending concentrations of alcohol and embedded in paraffin wax. From each gilt, three random slices were selected from the left ovary. These slices were then sectioned at 5μm, and every 50^{th} section placed on a microscope slide and stained with Harris hematoxylin and eosin. Follicles were counted as outlined by Griffin *et al* [115]. Follicular density was calculated by totaling all follicles in each category counted and divided by total volume.

2.4.6 Anti-Mullerian Hormone Assay

Blood (10ml) was collected from either the cranial vena cava or the brachiocephalic vein at Day 0, 15, 30, 45, and 60. Blood was centrifuged at 2000 rpms for 45 minutes. Serum was separated from the hematocrit and divided into 1mL aliquots

and frozen at -80°C until testing. Plasma concentrations of AMH were measured using a AMH ELISA kit (antibodies-online Inc., Atlanta, Georgia) according to manufactures instructions.

CHAPTER III

RESULTS

3.1 Preference Trials

3.1.1 Bait Consumption

To determine whether pigs would show a preference for a liquid or food-based bait, each pig was randomly assigned through four treatments. These treatments consisted of water, inactive FCB, water mixed with dry pig feed, and inactive FCB. Three observers rated each feedings session on a scale of 1-4, with one indicating high palatability and four as non-palatable. Pigs fed either water or liquid bait showed little to no interest when offered. However, palatability was significantly higher in pigs offered food-based treatments in comparison liquid treatments. (Figure 3.1). However, no significant difference was observed between the two liquid treatments as well as between the two food-based treatments.

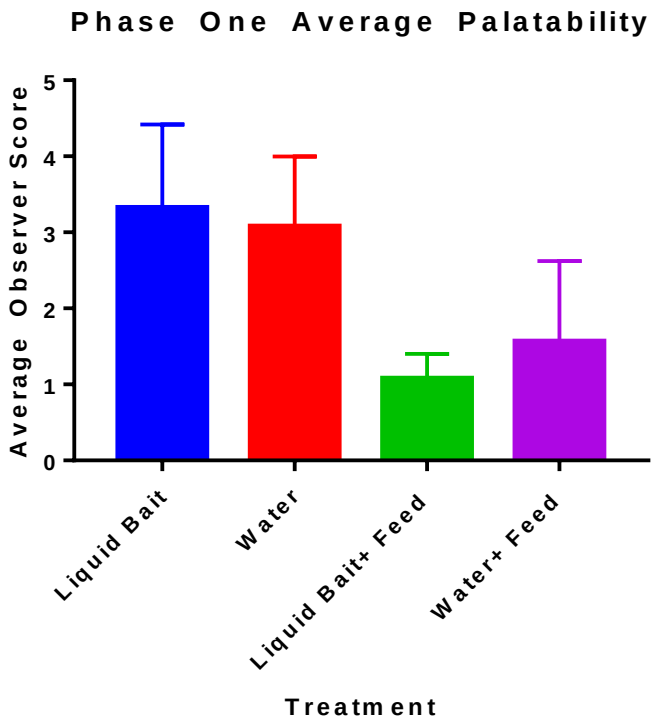

Figure 3.1 Palatability scores of liquid bait, water, liquid bait & feed, and water & feed during the preference trial. Average observation scores (n=120) were averaged for each of the four treatments. Palatability was observed on a 1-4 scale, with one indicating high palatability and four low palatability. ANOVA multiple comparisons.

3.2 Palatability Results

3.2.1 Inactive and Active Bait Consumption

To determine if the inclusion of triptolide and VCD in the FCB reduced consumption and caused changes in feeding behavior, six Sinclair pigs (three male and three female) were assigned an alternating feeding schedule. No significant difference was found between inactive and active bait (Figure 3.2). Five of six pigs consumed both

inactive and active FCB with no discernable difference in feeding behavior. One sow was initially observed consuming both treatment types with no observable changes until D8. From D8-14 consumption was erratic, with no observable association with alterations with treatment type (figure 3.3).

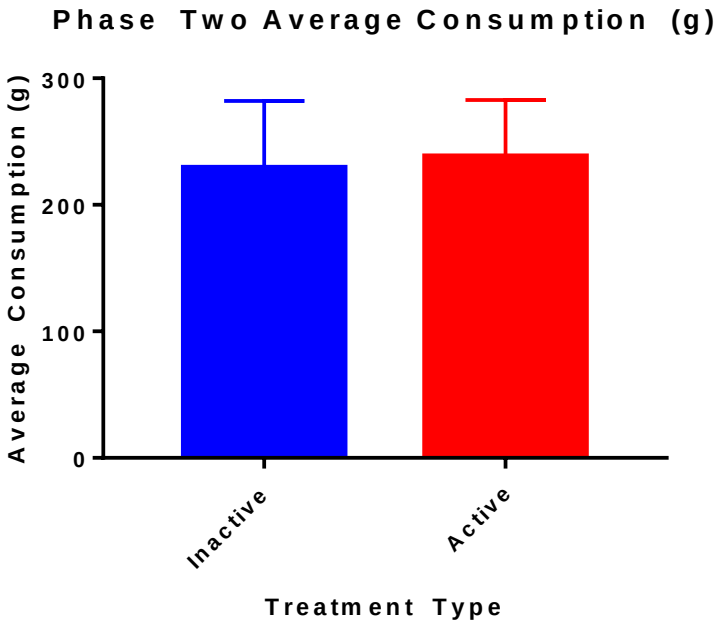

Figure 3.2 Consumption of inactive and active fertility control bait during the palatability trial. Average consumption of inactive and active fertility control bait during all feeding periods. Student *t*-test ($p>0.05$).

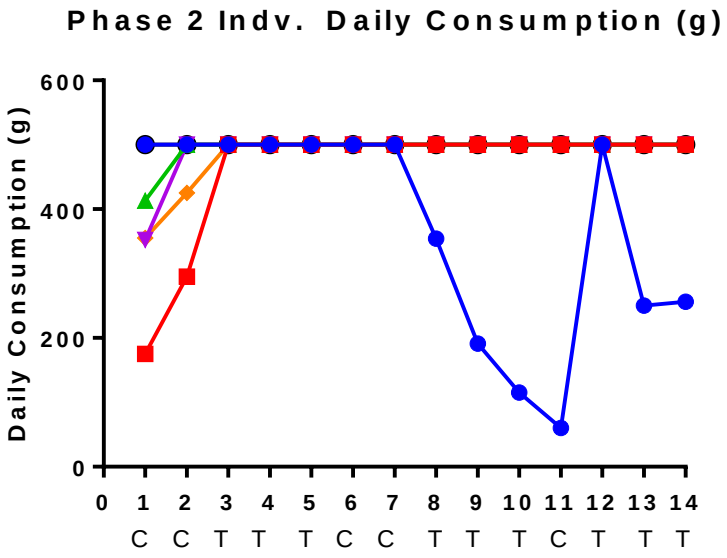

Figure 3.3 Individual daily consumption. Individual daily consumption (g) by each pig during the second phase. Inactive (C) and Active(T) indicate type of treatment received each day

3.3 Boar Efficacy Study

3.3.1 Bait Consumption and Active Dose

To determine if consumption of a FCB would decrease with prolonged consumption in boars, seven boars (four Sinclair and three commercials) were fed twice daily for 15 days the active FCB. Prior to the 15-day treatment period, pigs were fed inactive bait for five days. Commercial boars consumed entirety of bait during all feeding periods with no difference between inactive and active (figure 3.4). Sinclair boars consumed significantly more treatment bait than control bait (figure 3.5). Boar S3

consumed significantly less active bait in comparison to other boars (figure 3.6). Differences in consumption and weight resulted in variable dose of triptolide and VCD among individual boars (figure 3.6 and 3.7)

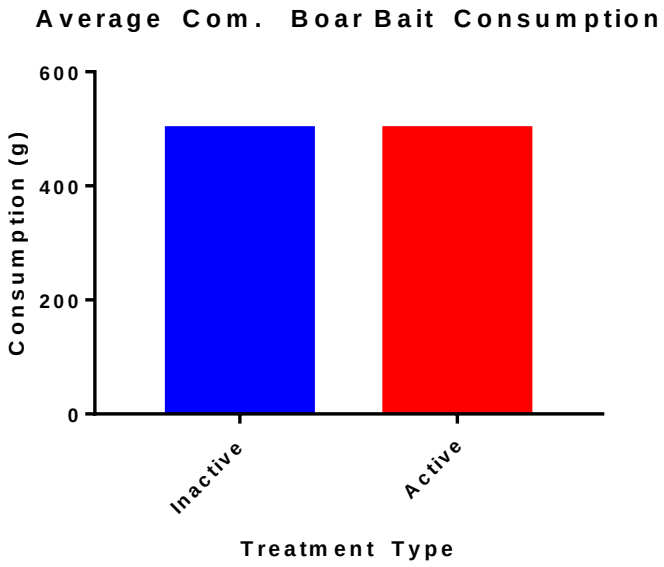

Figure 3.4. Consumption of inactive and active fertility control bait by commercial boar during the boar efficacy study. Average daily consumption of commercial (com.) boars between pre-bait

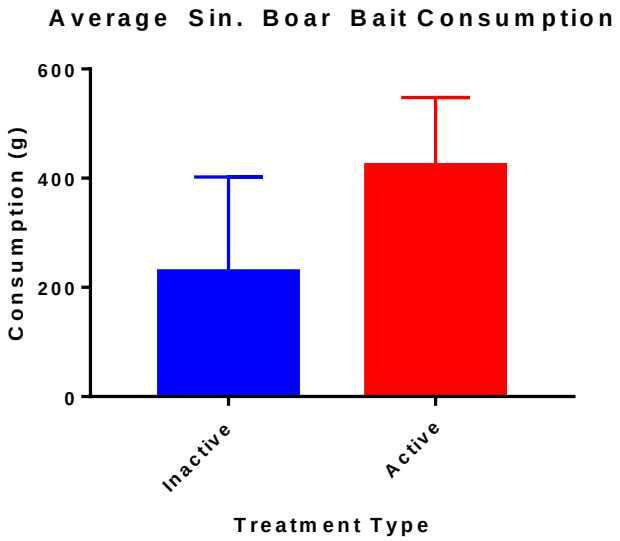

Figure 3.5 Consumption of inactive and active fertility control bait by Sinclair boar during the boar efficacy study. Average daily consumption of Sinclair (Sin.) between pre-bait (control bait) and

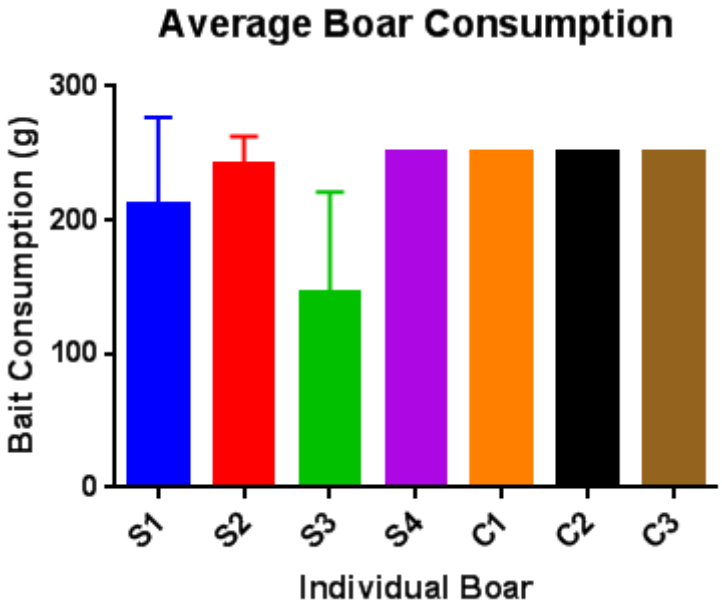

Figure 3.6 Individual boar consumption of active fertility control during the boar efficacy study. Average bait consumption of individual boar during each feeding period. ANOVA multiple comparisons.

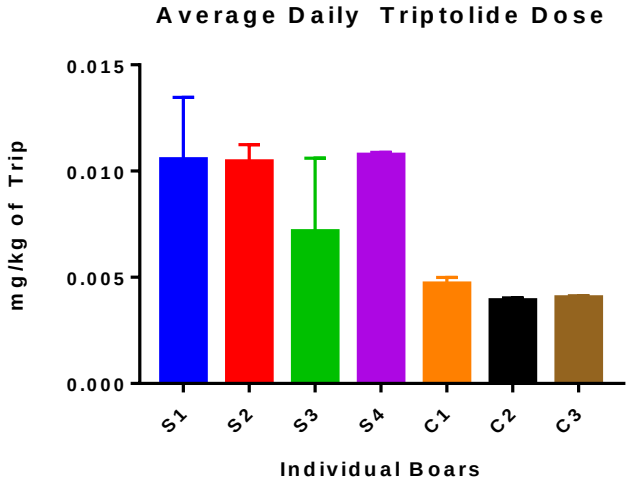

Figure 3.7 Average daily dose of triptolide received by each boar. Average dose (mg/kg) of triptolide consumed daily by each boar during the 15-day feeding period by Sinclair (S) and Commercial (C) boars.

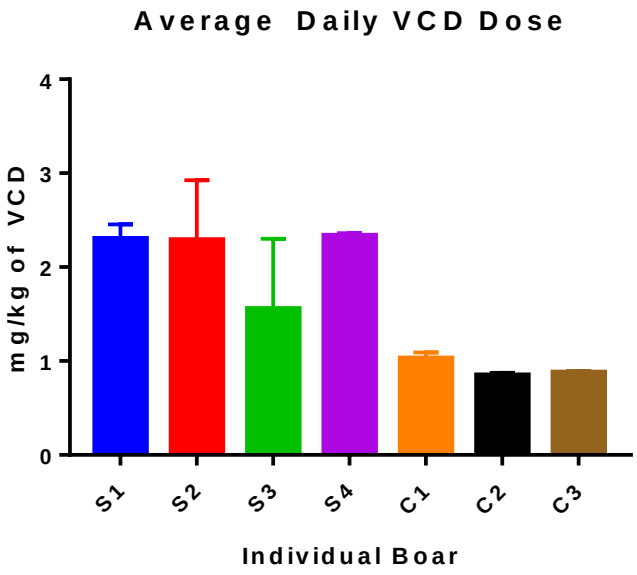

Figure 3.8 Average daily dose of VCD received by each boar. Average dose (mg/kg) of VCD consumed daily by each boar during the 15-day feeding period by Sinclair (S) and Commercial (C) boars.

3.3.2 Semen Viability

To determine if the FCB affected spermatozoa viability, spermatozoa collected at D0, 7, 15, 30, 37, 45, and 60 underwent viability staining. No significant reductions in viability were observed at D0,7, and 15. Significant reductions in average viability percentage were observed at D37, D45, and D60 in comparison to D0, 7, and 15 (figure 3.10). Two of five boars had less than 60% viability at D30 (figure 3.9). Four of five boars had less than 20% viability from D37-45. Prior to statistical analysis, boar S3

consumed reduced and inconsistent amounts of bait and was removed for statistical analysis (3.10).

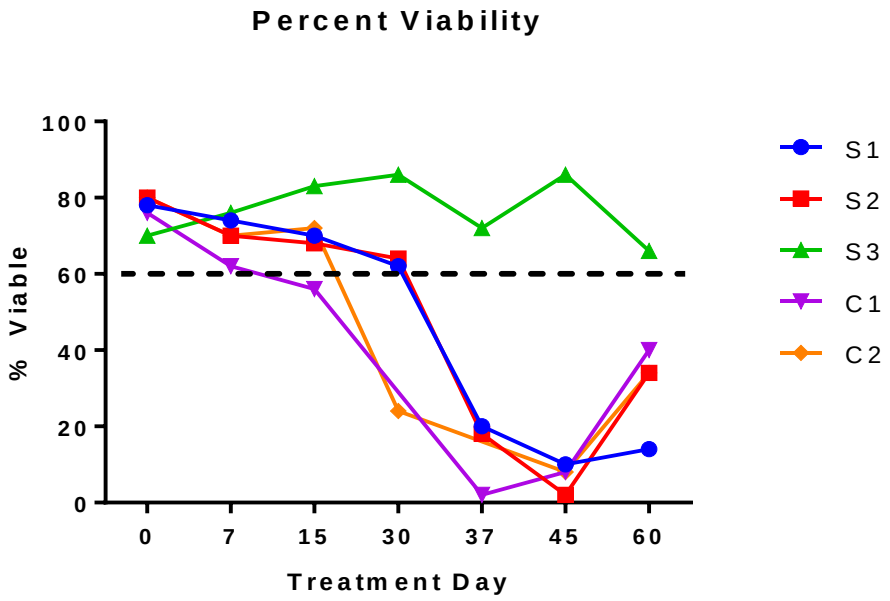

Figure 3.9 Individual viability scores at day 0, 7, 15, 30, 45, and 60. The percent viability observed at each time point for each boar. Values above the dotted line are considered normal

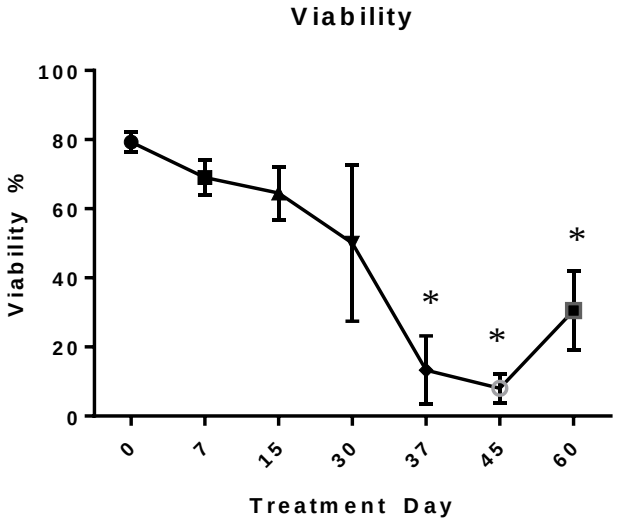

Figure 3.10 Average viability scores at day 0, 7, 15, 30, 45, and 60. Average viability percentage at each time point (n=4). Values above the dotted line are considered normal. Asterisks indicate significant reductions in viability in comparison to D0/7/15. Kruskal-Wallis ($p<0.05$).

3.3.3 Spermatozoa Morphology

To determine if the FCB affected morphology, spermatozoa were observed for abnormalities after nigorsin and eiosin staining. Morphological abnormalities were counted and categorized based on where the abnormality occurred as either head, neck, or tail abnormalities. Total abnormalities were calculated and expressed as a percentage of spermatozoa observed to have normal morphology. Prior to statistical analysis, boar S3 who consumed reduced and inconsistent amounts of bait was removed from statistical analysis (figure 3.6 and 3.7). No significant reductions in normal morphology was observed at D0,7, and 15. Significant reductions in normal morphology percentage

were observed at D37 and D45 in comparison to D0, 7, and 15 (figure 3.11). Sperm abnormalities at D37 and D45 largely consisted of decapitated sperm and abnormal midpiece causing folding onto the head region (figure 3.12)

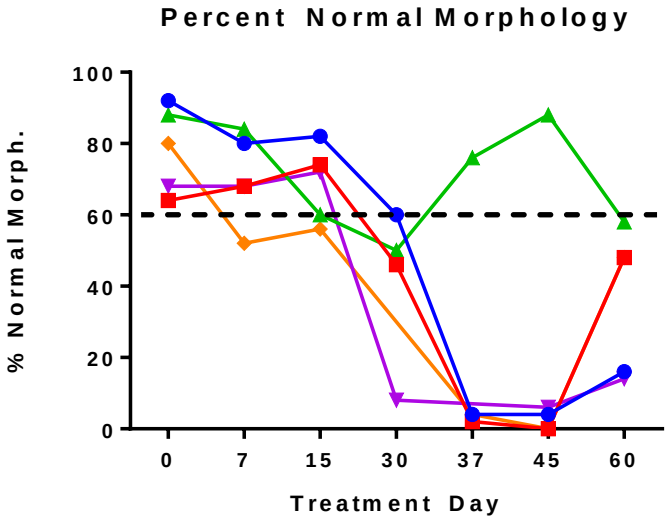

Figure 3.11 Individual normal morphology scores at day 0, 7, 15, 30, 45, and 60. The percent of spermatozoa with normal morphology observed at each time point for each boar. Values above the dotted line are considered normal

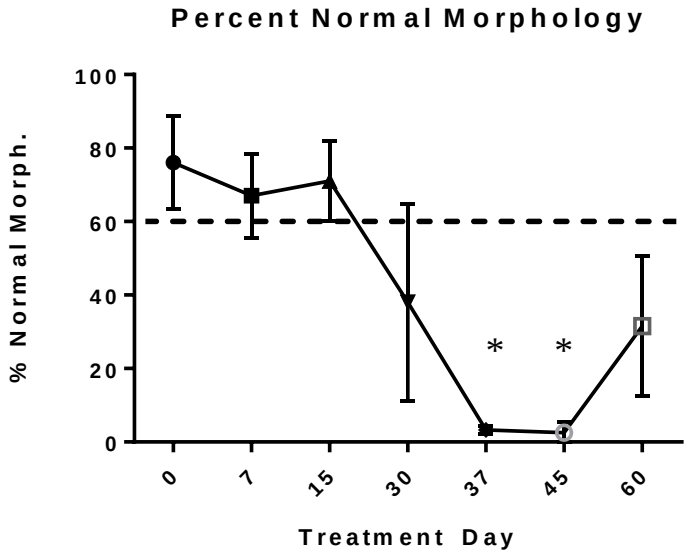

Figure 3.12 Average normal morphology at day 0, 7, 15, 30, 45, and 60. Average normal morphology percentage at each time point. Values above the dotted line are considered normal. Asterisks indicate significant reductions in morphology in comparison to D0/7/15. Kruskal-Wallis $p<0.05$.

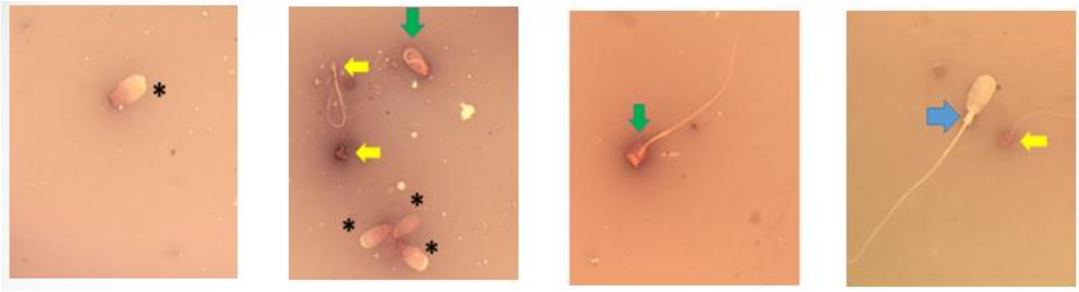

Figure 3.13 Morphological abnormalities commonly seen 30-45 days post treatment. Black asterisks (*) indicate tailless heads, downward green arrows indicate midpiece folding, blue arrows indicate midpiece swelling, and yellow arrows indicate decapitated spermatozoa

3.3.4 Progressive Motility

To determine if the FCB impacted progressive motility, wet mounts of fresh ejaculates were observed before, during, and after the study. Progressive motility (PM) was observed and rated on a 0-5 scale. PM values under 3 were considered subfertile [121]. PM in all boars was observed to be in normal ranges at D0, 7, and 15. Samples observed at D37 and D45 had little to no observable motility and high incidence of spermatozoa aggregating. One boar at D30 was observed to be subfertile. Four of five boars were subfertile from D37-60 (Figure 3.11). One boar that was observed to have consumed less FCB was not included for statistical analysis. Significant reduction in progressive motility was observed at D37 and D45 (Figure 3.13).

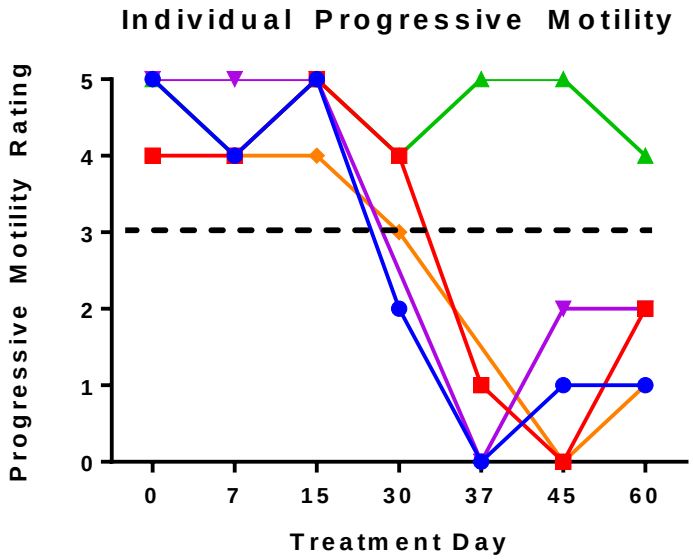

Figure 3.14 Individual progressive motility at day 0, 7, 15, 30, 45, and 60. Progressive motility of ejaculates scores observed at each time point for each boar. Values above the dotted line are considered normal.

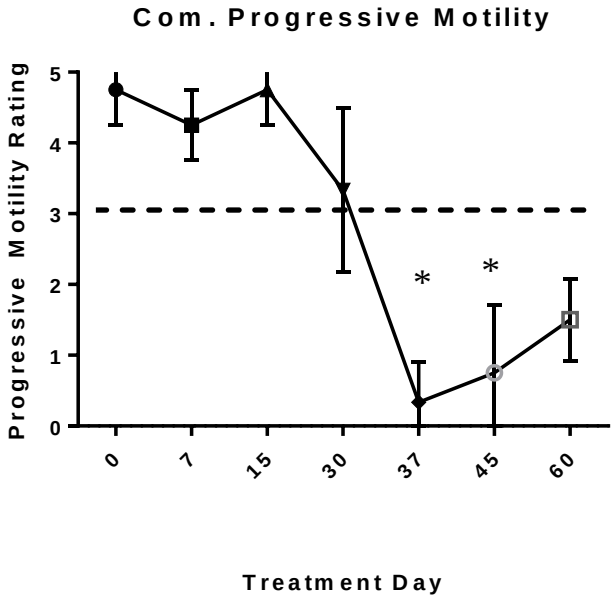

Figure 3.15 Average progressive motility at day 0, 7, 15, 30, 45, and 60. Average morphology percentage at each time point (n=4). Values above the dotted line are considered normal. Asterisks indicate significant reductions in PM in comparison to D0/7/15. Kruskal-Wallis $p<0.05$.

3.3.5 Semen Concentrations

To determine if the FCB reduced concentration of spermatozoa in collected ejaculates, freshly collected semen was diluted and counted using a hemocytometer. Spermatozoa in samples collected at D37 and D45 had high incidences of aggregating together, however no significant reductions were observed at any time point. Two boars were observed to trend downwards at D37 and D45 (Figure 3.13).

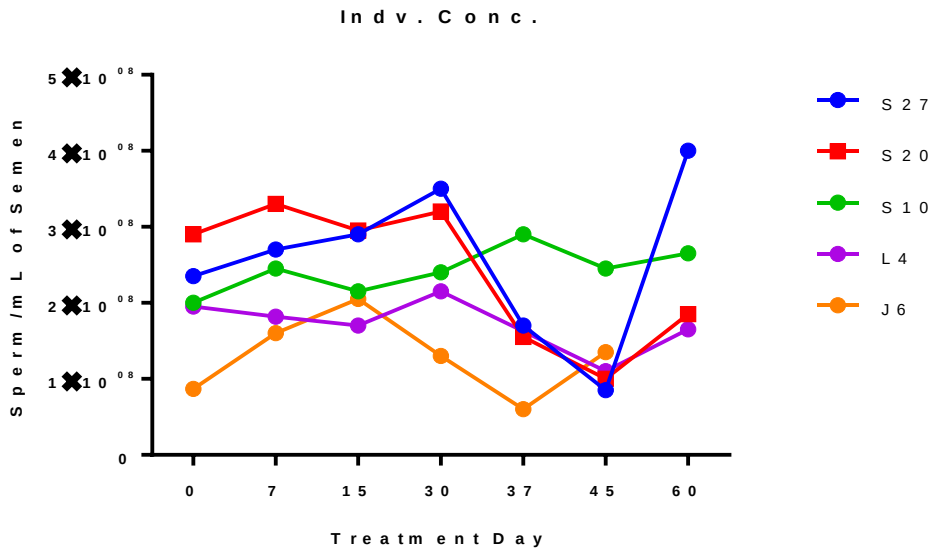

Figure 3.16 Individual seminal concentrations at day 0, 7, 15, 30, 45, and 60. Individual semen concentrations at each time point throughout the study.

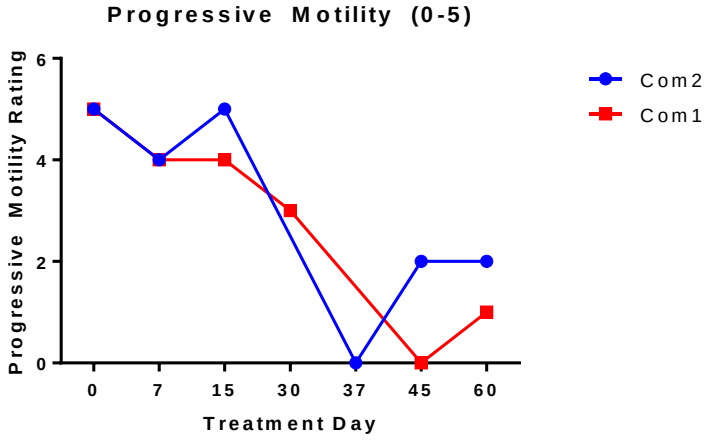

Figure 3.17 Average seminal concentrations at day 0, 7, 15, 30, 45, and 60. Average semen concentrations at each time point throughout the study. Kruskal-Wallis p>0.05.

3.3.6 Gonadosomatic Index

Gonadosomatic Index (GSI) was estimated throughout the study to determine if alterations in spermatogenic activity would manifest in changes in testes volume. No significant changes in GSI were observed at any time point (figure 3.18). One boar was observed to have reduced testes volume.

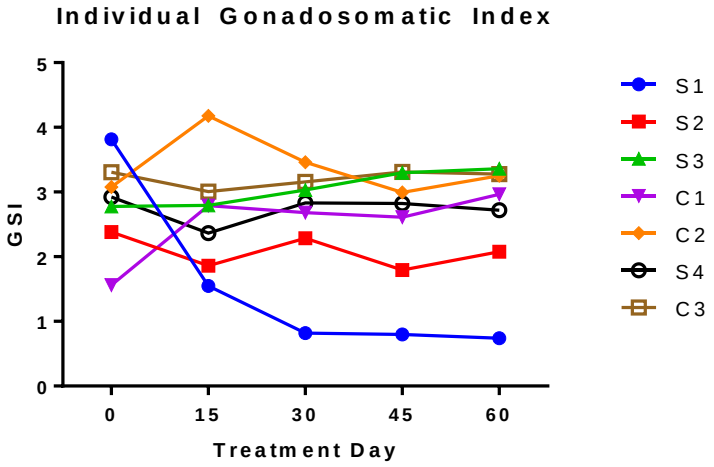

Figure 3.18 Individual boar gonadosomatic index at day 0, 15, 30, 45, and 60. Individual boar Gonadosomatic index (GSI) (testes volume (cm^3)/ bw (kg)) at each time point.

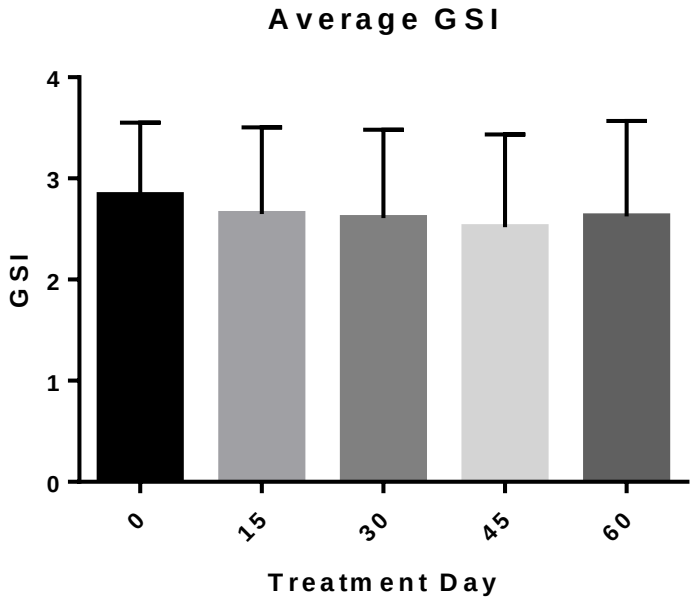

Figure 3.19 Average boar gonadosomatic index at day 0, 15, 30, 45, and 60. Average boar Gonadosomatic index (GSI) (testes volume (cm^3)/ bw (kg)) at each time point. Kruskal Wallis ($p>0.05$).

3.3.7 Plasma Testosterone Levels

To determine the effect of FCB on plasma testosterone levels, blood was sampled before, during, and after the active feeding period. No significant differences in plasma testosterone were observed (figure 3.19)

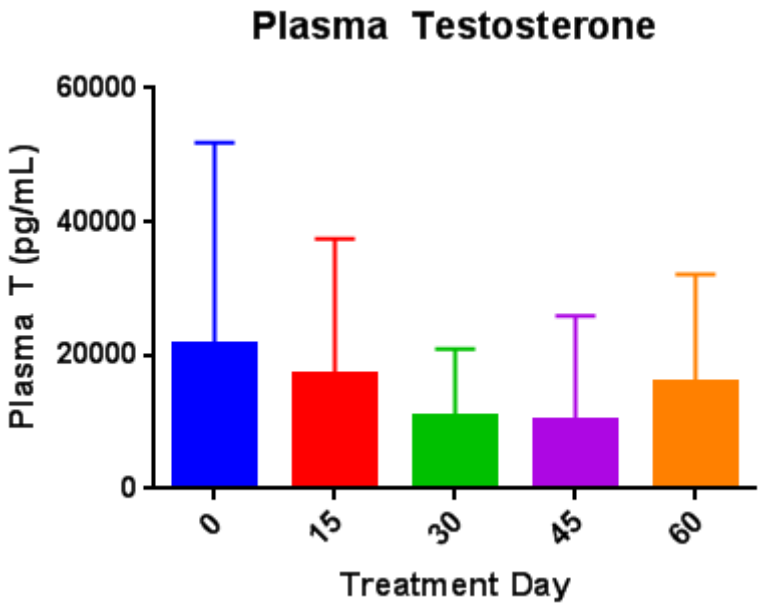

Figure 3.20 Testosterone levels at day 0, 15, 30, 45, and 60. Average plasma testosterone before and after the active feeding period. Kruskal Wallis ($p>0.05$).

3.3.8 Testes Histology

To determine the effects of the fertility control bait had on spermatogenic activity, testes sections were stained with hematoxylin and eosin. All but one boar had normal spermatogenic activity (figure 3.20).

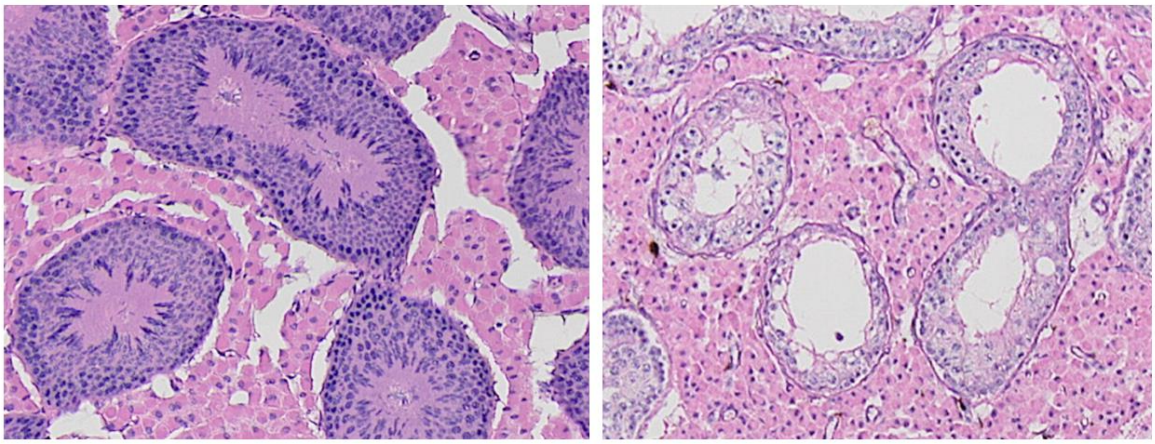

Figure 3.21 Seminiferous tubule morphology. Left, representative seminiferous tubules of boar at D60. Right, representative sample from seminiferous tubule from boar that did not have normal spermatogenic activity.

3.4 Sow Efficacy Study

3.4.1 Bait Consumption and Dose

To evaluate differences in consumption of the FCB, gilts were placed into control and treatment groups. All gilts consumed the entirety of bait at all time points throughout the feeding period. No significant differences in consumption between the two groups were observed (figure 3.16). One sow in the treatment group became ill and ceased consumption of both active bait and dry feed during the feeding period and was removed from the study.

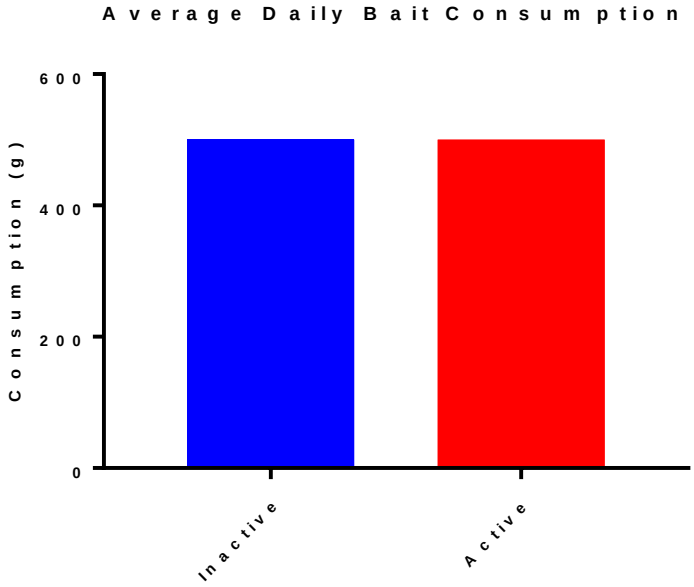

Figure 3.22 Gilt inactive and active bait consumption. Average daily consumption of inactive and active fertility control bait. Student *t-test* ($p<0.05$)

3.4.2 Ovarian Mass and Gonadosomatic Index

To determine if consumption of FCB caused a reduction in GSI indicative of decreases in the amount of large growing follicles, ovaries were removed after euthanasia and weights of left and right ovaries were summed and divided by bodyweight to calculate GSI. A significant reduction in GSI was observed between control gilts (18.94g) and treated gilts (8.648g) (figure 3.21).

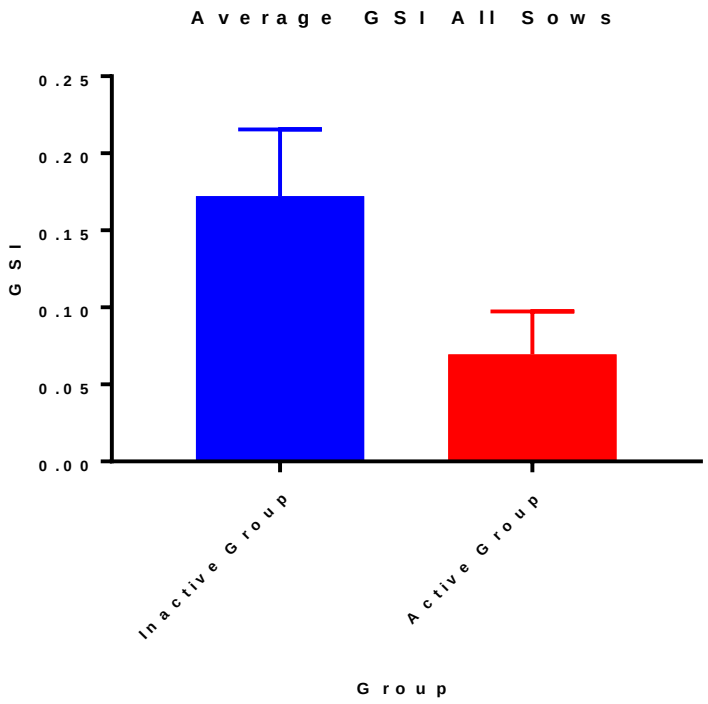

Figure 3.23 Gonadosomatic index of inactive and active gilts. Comparison of gonadosomatic index (GSI) between inactive and active gilts. Kruskal-Wallis ($p<0.05$).

3.4.3 Large Follicle Prevalence

To determine if the active feeding period reduced the number of observable growing follicles on the ovary, observable follicles were counted and measured using calipers. A significant reduction was observed in the amount of large growing follicles in treated gilts in comparison to control gilts (figure 3.22)

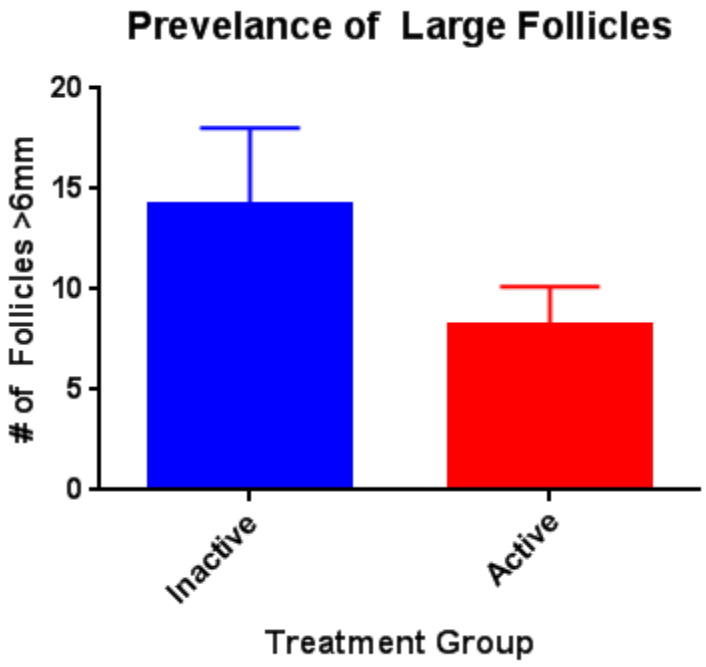

Figure 3.24 Prevalence of large antral follicles in inactive and active gilts. Comparison of average large follicle (>5mm) between inactive and active gilts. Kruskal-Wallis ($p<0.05$).

3.4.4 Primordial, Primary, and Secondary Follicular Densities

To determine if the active feeding period reduced the number of pre-antral follicles, random samples from the left ovary of each gilt were serially sectioned. Every 50th section was stained, counted, and volume calculated. The follicular density of each section was calculated by dividing the counts of each follicular type by the volume. A significant decline in the density of primordial follicles was observed in the active group (figure 3.23). Primary follicle density was also significantly reduced in the active group

(figure 3.24). A significant reduction in secondary follicle density occurred in the active group (figure 3.25).

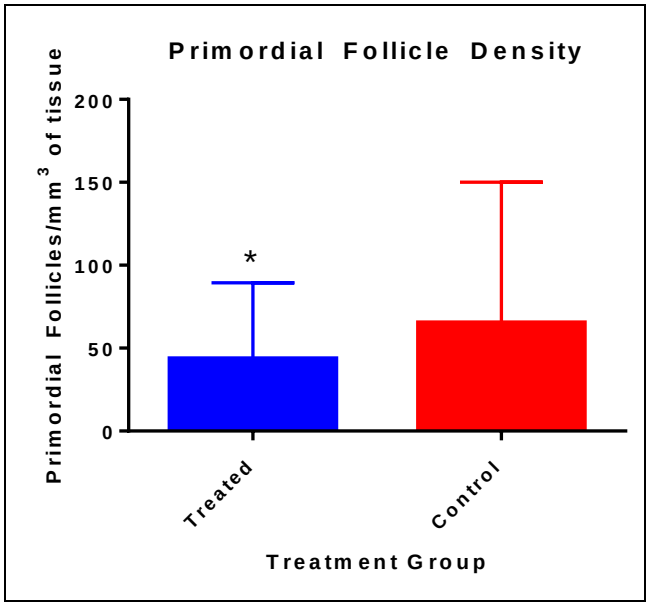

Figure 3.25 Primordial follicle density in inactive and active gilts. Comparison of primordial follicle density (primordial follicle per mm^3 of tissue) between inactive and active gilts. Student *t*-test ($p<0.05$).

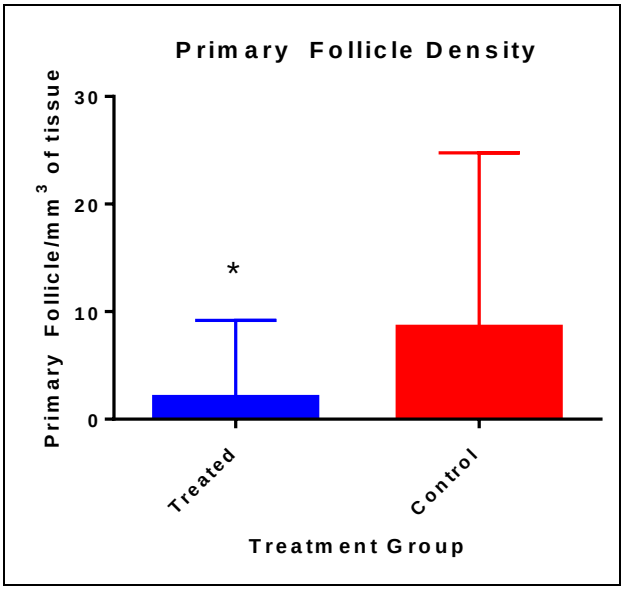

Figure 3.26 Primary follicle density in inactive and active gilts. Comparison of primary follicle density (primary follicle per mm^3 of tissue) between inactive and active gilts. Student *t*-test ($p<0.05$).

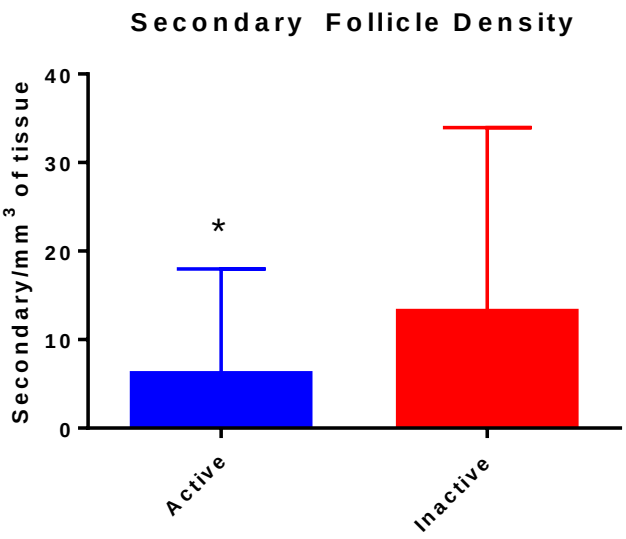

Figure 3.27 Secondary follicle density in inactive and active gilts. Comparison of secondary follicle density (primary follicle per mm^3 of tissue) between inactive and active gilts. Student *t*-test ($p<0.05$).

3.4.5 Anti-Mullerian Hormone Assay

To determine changes in serum AMH levels before and after the treatment period as well as in comparison to the inactive group, serum samples were run through a commercially available AMH ELISA kit. No significant changes were observed in the active group over time or in comparison to the inactive group at D60 (figure 3.26 and 3.27).

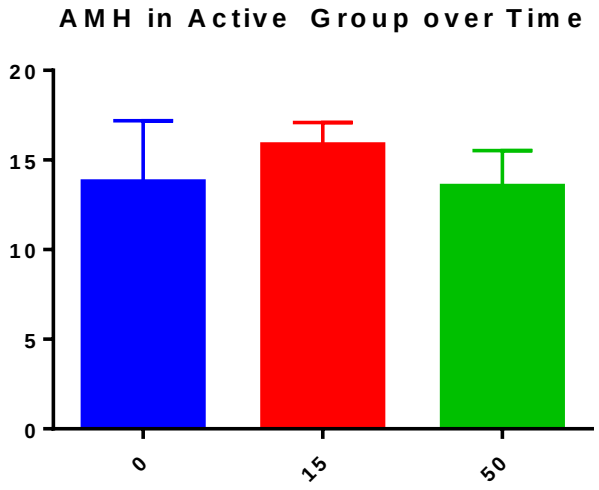

Figure 3.28 Serum Anti-Mullerian hormone level over time in active gilts. Comparison of serum AMH levels at D0, D15, and D50. Kruskal Wallis ($p>0.05$).

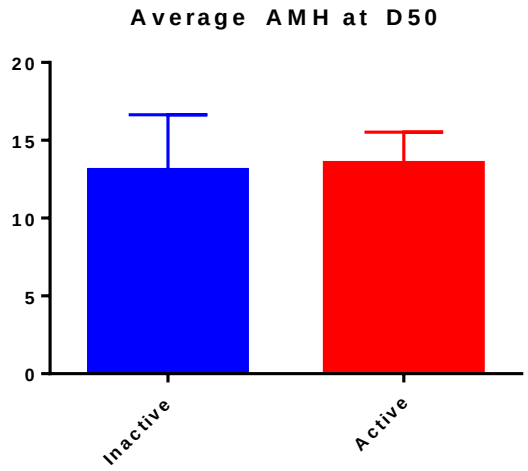

Figure 3.29 Serum Anti-Mullerian hormone level in active and inactive gilts. Comparison of serum AMH levels between active and inactive groups at D50. Kruskal Wallis ($p>0.05$).

CHAPTER IV

SUMMARY AND CONCLUSIONS

4.1 Palatability of Fertility Control Bait

For a FCB to be effective, high enough amounts of bait must be freely consumed for ingestion of efficacious doses. Previous studies established pigs preferred fat-rich and sweetened baits, which led to the development of the proprietary FCB implemented in these studies. The collective goal of this work was to evaluate the palatability of this FCB containing the active ingredients triptolide and VCD for evaluation of its impact on fertility in boars and gilts.

During the preference trials, it was demonstrated that captive pigs would prefer a food-based bait as opposed to a liquid. Pigs would either ignore or flip over bowl of water or bait, indicating little interest. When provided with a mixture of either pig feed and water or pig feed and inactive bait, pigs would readily consume both mixtures. This indicated that a food-based bait would be more palatable. There was no significant difference between the additions of water or the inactive bait indicating that both water and the bait were similarly palatable. It is possible that water and inactive bait presented without feed would have been more palatable if water was withheld from pigs for a longer period (>12 hours). As this would simulate scenarios encountered by wild pigs in arid regions where water is at a decreased prevalence.

Based upon the results of the initial trials, the bait underwent reformulation into a solid state. Strawberry flavoring was also added to the bait as it is a common feed additive for domestic pigs and was preferred in wild pig baiting studies [116]. The

second experiment was designed to evaluate differences in consumption and feeding behavior between inactive and active bait. Pigs were assigned to a feeding schedule that alternated between inactive and active bait. During this study, no difference was found between the amount of inactive and active bait consumed indicating that the presence of the active ingredients did not dissuade bait consumption.

One sow was found to decrease consumption of gelatin during the course of the study (figure 3.3). Coinciding with this, the sow also had decrease interest in pig feed (personal observation). It is possible that latent toxicity had caused aversion to consume bait regardless of type but that is unlikely due to the rebound in consumption of active bait on day 12. If toxicity had conditioned the sow to avoid consumption of bait, decreases in consumption of bait independent of bait type would have occurred. It is also possible that consumption of active bait had latent systemic toxicity causing the decrease in appetite from day 8-11. The decrease in consumption from day 8-11 may have caused the potentially toxic side effects to subside leading to an increase in health of the sow explaining the rebound in consumption seen at day 12. It is further possible that an illness unrelated to the study could have contributed to decreases in consumption of FCB and dry feed.

Interestingly, no difference in consumption of inactive and active bait occurred. This contradicts previous studies of a similar fertility control product for rats, where consumption decreased after the addition of triptolide and VCD [90, 117]. This suggests that pigs may have a decreased sensitivity to triptolide and VCD. Earlier investigations into a fertility control product for pigs using a similarly structured chemical as VCD,

ERL-4221, caused captive wild pigs to vigorously shake their head, excessively salivate, and perform body rolls on these baits indicative of an adverse oral reaction [89]. None of these behaviors were observed in any pigs during this study, suggesting that the inclusion of triptolide and VCD did not cause an adverse reaction during consumption. It is concluded that the sow with lowered consumption was an undiagnosed outlier, and that the reformulated FCB is a palatable bait suitable for efficacy studies.

During the boar efficacy study, active bait was found to be highly palatable over the course of the 15-day treatment period. Both forms of bait were readily consumed by commercial boars at all time points and was typically preferentially consumed before pig feed. Three of four Sinclair boars increased bait consumption over the course of the study independent of treatment type. Due to this neophobic response to a novel feed, consumption of control bait was significantly lower than in comparison to active bait (figure 3.5).

Boar S3 was observed to have inconsistent consumption of bait and dry feed throughout the study. If the bait was unpalatable to Boar S3 it would be expected that the boar would avoid the bait and consume only pig feed. This was not the case, as pig feed consumption was also inconsistent (personal observation). This suggests that the differences in consumption could be attributed to an individual difference in appetite as this boar also had variance in consumption of dry feed.

The difference in feeding behavior between commercial and Sinclair boars may be attributed to commercial breeds being artificially selected for higher growth rates that require increased appetites. The neophobic feeding behavior observed in Sinclair boars

bait could also be more reflective of consumption behavior by wild pigs as Sinclair pigs are recent descendants of North American feral pigs, indicating that future studies should also utilize inactive bait to acclimate pigs to the FCB.

During the sow efficacy study, active bait was highly palatable during the 15-day treatment period. In contrast to the boar study, no differences in consumption of occurred between the breeds. During all feeding periods, all but one gilt consumed the entirety of bait and as observed during the boar efficacy study, gilts would typically consume bait prior to pig feed. On the 8th day of the treatment period, a commercial gilt became lame with a fever (>40°C) and stopped consuming both bait and pig feed. Due to this, the commercial gilt was removed from the study and necropsy revealed the gilt was suffering from a *streptococcus* infection. It is unclear why no difference occurred between the breeds in gilts. It is possible that Sinclair boars have a pronounced neophobic response to a novel feed or bait in comparison to Sinclair gilts.

Overall, the results of these experiments demonstrated the FCB to be efficacious for use in further studies in pigs as it does not cause immediate or long-term toxicity in the animal. This study utilized domestic breeds due to availability and ease of handling. Although Sinclair pigs are recent descendants of feral pigs, future palatability studies should include wild pigs for a more accurate representation of consumption and feeding behavior. The FCB in its current form is limited for use in captive pigs and is unsuitable for application in field studies. At this time, the bait is solidified by gelatin that typically liquefies around 35 C°, which makes it unsuitable for field studies in most regions

inhabited by pigs. Previous studies indicated that baits with a paste consistency are more efficacious [50, 51] and future studies should test a paste form of this FCB.

Another important factor to consider for future formulations is wild pigs have a well-developed sense of smell and poor eyesight. Baits should be odorous and dyed a dark green color to decrease visual stimulation of non-target species [44].). Increasing the strawberry scent or additions of odorous scents such as a fish or peanut butter could increase palatability in field studies [113]. Although pigs have poor eyesight, studies have indicated that coloration of the bait alters consumption by wild pigs. Snow *et al* [51] showed success implementing a black peanut paste that microencapsulated sodium nitrite. This black peanut paste was artificially dyed black and was preferred over the naturally light brown peanut paste. Snow *et al* [51] suggested that this might be due to feral pig's natural behavior of rooting through dirt, which is typically darker brown or black in appearance. This indicates that the modified FCB may have been more palatable if altered from a bright pink to a more natural color.

Collectively, it is recommended that in future studies, FCB to be modified into a paste that will be more tolerant of variable weather conditions during field application. Additionally, increasing the prevalence of fat-rich flavors such as peanut butter or fish oil coupled with dying the bait black might increase palatability. Ultimately, the results of this study demonstrated that captive commercial and Sinclair pigs would freely consume a bait containing triptolide and VCD, and is a suitable for evaluation of its effects on pig fertility.

4.2 Boar Efficacy Study

The boar efficacy study was designed to evaluate the impact of the FCB on boar reproductive capacities. In males, fertility is dependent on the production of large numbers of functioning spermatozoa. For this bait to be effective in boars, it must disrupt either spermatogenesis within the testes or epididymal maturation significantly to result in reduced prevalence of functional spermatozoa. Collecting ejaculates prior to, during, and after the active phase allowed for monitoring of seminal parameters indicative of fertility. Semen was unable to be collected from two boars. From D0, the onset of this study to D30, no significant changes were observed in seminal parameters (figures 3.8-3.15). Twenty-two days after cessation of the active phase (D37), four of five boars had significantly reduced seminal parameters indicative of reduced fertility.

The viability stain allows for basic evaluation of the overall proportion of spermatozoa that were living or viable at the time of staining. Previous studies examining viability percentage in boars found insignificant variation between low and high fertility boars [118]. It is important to note that while a spermatozoon may be deemed viable it does not elucidate on its motility and fertilization capabilities. From D0 to D15, no changes in viability had occurred, but at D30 one boar experienced a fifty percent drop in viability suggesting a decrease in fertility.

The ability for a spermatozoon to swim in a linear direction or to be progressively motile is crucial for it to be able to navigate the female reproductive tract and complete fertilization. A portion of each semen sample was observed and rated on a 0-5 scale [119]. PM can be used as method to estimate potential boar fertility as previous

studies have observed that boars with low fertility (<20% farrowing rates) averaged a PM score of 3 boars [118]. As seen in the viability stain, no significant changes were observed in PM from D0-D30, while boar C2 at D30 possessed a subfertile PM score of two.

Another important attribute for spermatozoa to possess is to have low numbers of morphological abnormalities as they can greatly affect viability and motility. The proportion of morphologically normal spermatozoa can serve as indicator of fertility as high fertile boars (80-100% farrowing rates) average 90% morphologically normal spermatozoa while low fertility boars had 60% percent of spermatozoa with normal morphology [118]. From D0-D30, only boar C2 at D30 was observed to have a normal morphology percentage under 60%.

The boar is constantly producing large quantities of spermatozoa. Reductions in production result in decreases in concentration of spermatozoa within seminal fluid, which can influence fertility. Larsson [119] indicated that normal semen concentrations in boars should be greater than 10^7 spermatozoa per mL of seminal fluid. Further, changes in concentration indicate disruptions in either spermatogenesis or epididymal maturation. As seen in the previous parameters, no decline in seminal concentration was observed from D0-D30.

The lack of change in any of these parameters at any time points indicated that epididymal maturation was not immediately affected. During epididymal maturation, the cytoskeletal structure of the spermatozoa completes development and becomes motile. If immediately disrupted, it would be expected to observe reductions in seminal parameters

at D7 or D15 as spermatozoa collected at D15 completed the entire process of epididymal maturation during the active phase. This contradicts previous studies in rats, that after seven days of intraperitoneal injections of triptolide (60μg/kg) seminal concentrations decreased by over fifty percent indicating disruption of epididymal maturation.

The mechanism behind triptolide affecting epididymal maturation has not been established. It is most likely due to the generation of ROS during triptolide metabolism as observed in the kidney, liver, and ovarian follicles [101, 120]. During spermatogenesis and epididymal maturation, reactive oxygen species (ROS) are natural by products of these processes [121]. To compensate, the epididymis possesses an extensive antioxidant system to prevent the accumulation of ROS leading to oxidative stress [122]. The plasma membrane of spermatozoa is made up of considerable amounts of polyunsaturated fatty acids making it highly susceptible to perioxidation from ROS [121]. Inducing oxidative stress in boar spermatogenesis with lipopolysaccharide, resulted in similar abnormalities in spermatozoa observed in previous studies with triptolide treated male rats [104, 123]. Collectively, this suggests that increasing the dose of triptolide would lead to an increased accumulation of ROS within the epididymis, resulting in disruption of the maturation processes.

At D37 and D45, four of five boars were observed to have less than 20% viable sperm. Due to this low proportion of spermatozoa potentially capable of completing fertilization between D37 and D45, it is suggestive of a decrease in fertility at these time points. Similar reductions in PM in these four boars occurred, as samples collected at

these time points typically consisted of large aggregations of immobile spermatozoa. Variations in PM between studies is a concern as it is subject to technician variation, however the large aggregations of immobile sperm that were prominent at D37 and D45 would have received substantially lower scores in comparison to samples collected at D0,7, and 15 regardless of technician. Further, the PM scores at D37 and D45 were 0.33 and 0.75, respectively, are well below observed scores in other low fertility boars.

The observed decreases in viability and PM could be attributed to the large proportion of abnormal spermatozoa also observed at D37 and D45, as abnormal spermatozoa are unlikely to be viable or motile. At D37 and D45, four of five boars were observed to have less than 20% normal spermatozoa indicating fertility had been reduced. Severe abnormalities such as head-tail separation were commonly observed at these time points. These morphological abnormalities are similar to previous studies examining triptolide's effect on the male reproductive system in rats [103, 104]. It is unclear at what point these decapitations occurred. It is possible that abnormalities occurred during spermatogenesis or epididymal maturation, and resulted in a weakened structure that manifested during the stresses of ejaculation or evaluation.

The other commonly observed morphological abnormalities were swollen midpiece and weakened midpiece attachment, which resulted in the folding over of the midpiece onto the head. The swollen midpiece indicates abnormal mitochondrial sheath assembly. Improper formation of the mitochondrial sheath has been observed to render spermatozoa susceptible to decapitation and folding [124]. This implicates the abnormal

mitochondrial sheath is causing the decapitations of spermatozoa and folding of the midpiece.

In contrast to the previous parameters, only two of five boars were observed to have large reductions in seminal concentrations at D37 and D45. During spermatogenesis and epididymal maturation, defective spermatozoa undergo an ubiquitination process, marking them for reabsorption or phagocytosis by macrophages within the epididymis [125]. It is unclear why this process would not have caused a significant reduction in concentration, as large amounts of non-functioning spermatozoa observed in this study would certainly be candidates for ubiquitination. It is possible that due to the relatively young age (6-12 months of age) of these boars, that sperm production could still have been increasing but triptolide and VCD slowed this growth resulting in the observed plateaus (figure 3.15 and 3.16).

As noted, boar S3 was found to have no significant variation in any seminal quality parameters throughout the study. Boar S3 consumed inconsistent and reduced amounts of bait in comparison to the other boars. Interestingly, due to the smaller size of this boar, it was receiving a similar dose (mg/kg) of triptolide and VCD. Previous studies, observed that triptolide accumulated in the testes of rats [126] suggesting that the dose of triptolide that induces deleterious effects on seminal quality may not be weight dependent.

Boar S3 was removed from statistical analysis due to reduced consumption. Upon removal of the outlier, the observed reductions in viability at D37 and D45 were found to be statistically significant in comparison to D0, 7, and 15. Further, the

reductions in PM and morphology were also significantly reduced at D37 and D45. In contrast, no significant changes were observed in seminal concentration were observed. Reductions in seminal parameters occurred at D30 in boar C2, while in the remaing boars at D37 indicate perturbation most likely occurred between D30 and D37. The timing of this onset, suggests the inhibition of latter stages of spermatogenesis had occurred.

It is possible that the breakdown in spermatogenesis was the result of decreased testosterone stimulation. Leydig cells, producers of testosterone, have been shown in *in vitro* studies to be susceptible to triptolide triggered apoptosis [106]. No significant reductions in plasma testosterone levels were observed, indicating that Leydig function and the endocrine system was not responsible for disruptions in spermatogenesis. The lack of change in testosterone observed may have been due to the decrease dose of triptolide used in this study as rats receiving injections of 60μg/kg of triptolide for seven days had reduced plasma testosterone levels suggesting Leydig cell function was decreased [107]. In short, it is unlikely that testosterone played a role in the reductions in seminal parameters.

Recently, it was observed that glutathione perioxidases (GP-x4) expression in the testes were reduced in male rats after oral gavage of triptolide (0.1mg/kg for 30 days) [127]. In infertile men, GP-x4 expression is found to be significantly reduced in spermatozoa [128]. Additionally, a structural component of all GP-x4 variants is selenium and similar midpiece abnormalities as seen in this study are observed in selenium deficient mammals [129]. During metabolic breakdown of triptolide, the epoxide rings are

conjugated by glutathione and is catalyzed by GP-x4 [130]. Within developing spermatocytes and spermatids, three different forms of GP-x4 are expressed, with mitochondrial GP-x4 (mGP-x4) the most prevalent [131]. mGp-x4 not only serves in aiding in the metabolism of xenobiotic compounds such as triptolide but also plays a significant role in mitochondrial sheath structure [132].

Specifically knocking out mGP-x4 in mice, resulted in reductions in motility and an increase in morphological abnormalities such as swelling and folding of the midpiece. Additionally, no decreases in spermatogenic activity occurred suggesting mGPx-4 only affects mitochondrial structure of the spermatozoa. Spermatids just prior to spermiation, in both triptolide treated rats and mGP-x4 depleted mice appeared morphologically normal, but upon reaching the epididymis, folding and disfiguring of the midpiece occurred [133]. Collectively this suggests that in this study, that repeated exposure to the FCB depleted the GP-x4 within the testes resulting in incomplete midpiece assembly and is responsible for the folding and decapitated spermatozoa observed.

Interestingly, at D60, viability was still significantly reduced yet all other seminal parameters began to rebound. This suggests that seminal quality may have also been rebounding. Both triptolide and VCD have been found to reduce spermatogenic activity. In particular, VCD reduced the prevalence of proliferating spermatogonia and spermatocytes in rats. If this had occurred here, it would be expected that the disruption in early stages of spermatogenesis would manifest in reduced seminal concentration by D60.

Widespread decreases in spermatogenic activity would have reduced diameters of seminiferous tubules, which may manifest in reduced testes volume. No significant decreases in testes volume was observed. It is possible that reductions in spermatogenic activity would only affect testes mass and not volume. Additionally, the small sample size of this study coupled with measuring testes on live animals may have decreased accuracy of the results. It is possible that increasing the dose of VCD would disrupt early spermatogenesis that would cause a marketable decrease in concentration around D60. Pigs in this study were receiving 1-2mg/kg of VCD for 15 days which is considerably lower in comparison to the 320mg/kg r of VCD received by rats daily for 30 days. Overall, the lack of change in testes volume and concentration suggest early stages of spermatogenesis were largely unaffected.

Ideally, the effects of this FCB bait would be permanent or last at least for one breeding season. Currently, these data suggest a transient period of subfertility for approximately 14-21 days. It is possible that increasing the dose of triptolide and VCD would prolong these effects an increased dose of triptolide may disrupt epididymal maturation, accelerating the onset of reduced seminal parameters. Further, increasing the dose of VCD may prolong the effects of the FCB due to the reduction in early spermatogenesis stages. Collectively this could lead to extending these effects for at least two months. Inducing subfertility for two months would allow for distribution of FCB during known peak breeding times in wild pig populations.

Ultimately, the results of the boar efficacy study show considerable promise as it is unlikely that four of the five boars from D37 and to D45 would have been fertile.

Although not significantly reduced at D60, the results suggest that these boars may have still been sub fertile. Supplemental work is needed to examine higher doses of the active ingredients as well as changes in farrowing and litter rates as this study only examined seminal quality as an indicator of fertility. Although increasing the dose may hinder the palatability of FCB. It is recommended that in conjunction with investigating varying doses of triptolide and VCD that palatability of these new modified baits is also investigated.

4.3 Sow Efficacy Study

The gilt efficacy study was designed to evaluate the impact of the FCB on the reproductive functions of cyclic gilts. In the female pig, fertility is dependent upon the production of viable oocytes. For this bait to be effective in gilts, it must successfully disrupt folliculogenesis to reduce the amount of ovulated oocytes, thereby decreasing potential litter size. To evaluate the efficacy of the FCB in gilts, synchronized cyclic gilts were placed into active and inactive groups. At the time of euthanasia, a significant reduction in GSI and the prevalence of large antral follicles (>5mm) were observed in active gilts. Further, histological examination of ovaries found a decline in the frequency of primordial, primary, and secondary follicles in active gilts. In contrast, no difference in AMH level was found between the two groups.

At the end of the study (D50), all gilts were in the follicular phase of the estrous cycle. The morphology of the ovary during this phase is characterized by the presence of large (>5mm) preovulatory follicles preparing for ovulation and reductions in smaller follicular stages. The overall amount of large follicles present at this time contributes

significantly to GSI, which can be used as an indicator for the amount of large antral follicles as antral spaces contribute a significant portion of ovarian mass. In the active group, GSI was significantly reduced (value) in comparison to the active group suggesting that a decrease in the amount of preovulatory follicles.

This was confirmed by enumerating and measuring the large surface follicles. The average amount of follicles observed in the active group was significantly lower in comparison to the inactive group. This suggests a decrease in fertility in the inactive group as a reduced number of oocytes would have been available for ovulation and fertilization. King and Williams [134] developed a regression model to estimate litter size based off of ovulation rate (OR) and found for every ovum ovulated, litter size increased by 0.3 piglets for each ovulated ova. Assuming all antral follicles ovulated and none underwent atresia, the estimated average litter size in the active group of 7, a modest reduction in comparison to the average litter size of the inactive group (average litter size of 9). Although it is possible a greater proportion of these follicles in the active group were atretic.

From initial primordial activation to ovulation on average takes 116 days in pigs [77]. Due to this, pre-ovulatory follicles observed at euthanasia were already at the secondary change during the active treatment period, indicating these follicles were susceptible only to triptolide-induced atresia. Prior to D50, sows came into estrous during the late stages of the active phase (approximately D12) and approximately 18 days after cessation of treatment (D33). Due to the experimental design, it is unclear if the previous OR of the previous estrous periods would have been higher or lower as it is

unclear at what developmental stage ovarian follicles are most susceptible to triptolide. In either case, at D50 the reduced preovulatory follicles observed is suggestive of a reduction in fertility.

During histological evaluation, a similar reduction in density of secondary follicular was also observed. After activation, primordial follicles take on average 50 days to reach the secondary stage, which indicates that secondary follicles observed at D50 became activated during the initial days of the treatment period. The decrease in secondary follicular density observed suggests that the FCB reduced the activation rate of primordial follicles. Further, as these secondary follicles will need another approximately 65 days to complete maturation the difference in secondary follicular density would most likely manifest as a continued reduction in OR over this time period. In short, this suggests that the effects of the FCB would be prolonged past D50.

In conjunction with decreased secondary follicular density, a significant reduction in primary follicles in the active group occurred. Based on the kinetic growth rate of porcine folliculogenesis, primary follicles at D50 underwent activation around D20 of this study [77]. This suggests the primordial follicle activation continued inhibition of primordial activation after cessation of the active phase. Further this would indicate a continued decrease in OR in continued estrous cycles. Collectively, these reductions in follicular waves, suggest the effects of the FCB to continue for approximately another hundred days.

Ideally, this FCB would result in a permanent decline in fertility. As the ovary is unable to replenish its primordial follicle pool, complete elimination of all primordial

follicles would render gilts infertile. Here, it was observed that a significant reduction in primordial follicular density occurred within the active group. Although significantly reduced, the persistence of primordial follicles nests within the ovaries of gilts in the active group suggest a biologically insignificant depletion occurred. Due to the incomplete depletion observed in this study, it is possible that with time, folliculogenesis in active gilts would rebound to rates observed in the inactive group.

Within the active group, no significant reductions in AMH were observed during the course of this study. Further, in comparison to the inactive group, no reductions in AMH occurred. This contradicts the literature as previous experiments found dosing rats or hamsters with VCD resulted in reduced AMH levels [135, 136]. AMH acts to inhibit recruitment and serves as an indicator of follicular reserves in a number of species [137]. The lack of change in AMH suggests that the modest decreases in primordial follicle reserve were insignificant. Although it was been previously suggested that AMH in the pig ovary has a different mechanism of action than observed in humans and rodents [138]. The results observed here may support this as a decrease in primary follicles would be expected to lower AMH levels, but was not the case. In either case, the histological evidence and AMH assay suggest no significant effect on ovarian follicular reserve.

Ideally, the FCB would induce permanent reductions in fertility or at least last for one breeding season. The modest reductions in follicular development suggest a slight decrease in fertility of the active gilts indicate neither of these were achieved. In this study, pigs were receiving doses of triptolide and VCD. The doses used here are

considerably lower than in previous studies with rat containing 1% VCD and 200μg of triptolide and VCD, respectively [90] This indicates a higher dose of triptolide and VCD may increase the reductions of all follicular stages observed here. In theory, if an increased dose caused a larger majority of antral, secondary, and primary follicles to undergo atresia, a significant reduction in fertility would occur for approximately four months. Due to the length of time of it takes a follicle to complete folliculogenesis; this has a major impact on deciding when to distribute baits to wild pigs.

In wild pigs, reproduction varies with the environment. Wild pigs inhabiting colder regions, experience decreased reproductive functions during the winter months, as such ovarian activity declines due to the decreases in gonadotropin stimulation. Due to this, distribution of FCB to wild pigs during these times would only be targeting follicular reserve. It is important to note that the FCB would still act to decrease ovarian follicular reserve, as Roosa *et al* [135] found no differences in follicle depletion in Siberian hamsters dosed with VCD during short (winter) and long (summer) day lengths. It would be efficacious to time distribution of FCB just prior to typical peaks of breeding to decrease both developing follicles and follicular reserve. Ultimately, the results indicate further investigation into higher doses of triptolide and VCD is needed to investigate if the that leads to a greater decline in folliculogenesis and follicular reserve and what effects on fertility that causes.

REFERENCES

1. (USDA) USDOA. 2015: Questions and Answers: APHIS National Feral Swine Damage Management Environmental Impact Statement. In: USDA, editor. Washington DC2015.

2. Mayer J. Estimation of the number of wild pigs found in the United States. SRNL-STI-2014-00292. Savannah River National Laboratory, Aiken, SC, 2014.

3. Bengsen AJ, Gentle MN, Mitchell JL, Pearson HE, Saunders GR. Impacts and management of wild pigs Sus scrofa in Australia. Mammal Review. 2014;44(2):135-47.

4. Long JL. Introduced mammals of the world: their history, distribution and influence: Csiro Publishing; 2003.

5. West P. Assessing invasive animals in Australia. National Land and Water Resources Audit, ACT. 2008.

6. Deberdt AJ, Scherer SB. O javali asselvajado: ocorrência e manejo da espécie no Brasil. Natureza & Conservação. 2007;5(2):23-30.

7. Pedrosa F, Salerno R, Padilha FVB, Galetti M. Current distribution of invasive feral pigs in Brazil: economic impacts and ecological uncertainty. Natureza & Conservação. 2015;13(1):84-7.

8. Pimental D. Environmental and economic costs of vertebrate species invasions into the United States. Managing Vertebrate Invasive Species. 2007;38.

9. Tolleson DR, Pinchak WE, Rollins D, Hunt LJ, editors. Feral hogs in the rolling plains of Texas: perspectives, problems, and potential. Great Plains Wildlife Damage Control Workshop Proceedings; 1995.

10. Mazzoni della Stella R, Calovi F, Burrini L. The wild boar management in a province of the central Italy. Journal of Mountain Ecology. 2014;3.

11. Cummings K, Rodriguez- Rivera L, Grigar M, Rankin S, Mesenbrink B, Leland B, et al. Prevalence and Characterization of Salmonella Isolated from Feral Pigs Throughout Texas. Zoonoses and Public Health. 2016; 63:436-441

12. Baroch JA, Gagnon CA, Lacouture S, Gottschalk M. Exposure of feral swine (Sus scrofa) in the United States to selected pathogens. Canadian Journal of Veterinary Research. 2015;79(1):74-8.

13. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17(1).

14. Jay MT, Cooley M, Carychao D, Wiscomb GW, Sweitzer RA, Crawford-Miksza L, et al. Escherichia coli O157: H7 in feral swine near spinach fields and cattle, central California coast. Emerg Infect Dis. 2007;13(12):1908-11.

15. Gaskamp JA, Gee KL, Campbell TA, Silvy NJ, Webb SL. Pseudorabies Virus and Brucella abortus from an Expanding Wild Pig (Sus scrofa) Population in Southern Oklahoma, USA. Journal of Wildlife Diseases. 2016;52(2):383-6.

16. Hahn EC, Fadl-Alla B, Lichtensteiger CA. Variation of Aujeszky's disease viruses in wild swine in USA. Veterinary Microbiology. 2010;143(1):45-51.

17. Wyckoff AC, Henke SE, Campbell TA, Hewitt DG, VerCauteren KC. Movement and habitat use of feral swine near domestic swine facilities. Wildlife Society Bulletin. 2012;36(1):130-8.

18. Ickes K, Dewalt SJ, Thomas SC. Resprouting of woody saplings following stem snap by wild pigs in a Malaysian rain forest. Journal of Ecology. 2003;91(2):222-33.

19. Mungall EC. Exotics. Ecology and Management of Large Mammals in North America Prentice Hall, Upper Saddle River, NJ, EUA. 2000.

20. Sweitzer RA, Van Vuren DH. Rooting and foraging effects of wild pigs on tree regeneration and acorn survival in California's oak woodland ecosystems: USDA Forest Service Gen. Tech. Rep., Grand Forks, ND; 2002.

21. McCreary DD. Native oaks: the next generation. Fremontia. 1990;19(3):44-7.

22. Pirożnikow E. The influence of natural and experimental disturbances on emergence and survival of seedlings in an oak-linden-hornbeam (Tilio-Carpinetum) forest. Polish Journal of Ecology. 1998;46(2):137-56.

23. Singer FJ, Swank WT, Clebsch EE. Effects of wild pig rooting in a deciduous forest. The Journal of wildlife management. 1984:464-73.

24. Fordham D, Georges A, Corey B, Brook BW. Feral pig predation threatens the indigenous harvest and local persistence of snake-necked turtles in northern Australia. Biological Conservation. 2006;133(3):379-88.

25. Lewis TE, Atencio D, Butgereit R, Shea SM, Watson K, Keinath J, et al., editors. Sea turtle nesting and management in northwest Florida. Proceedings of the fifteenth

annual workshop on sea turtle biology and conservation Keinath, JA, DE Bernard, JA Musick, and BA Bell, editors NOAA Technical Memorandum NMFS-SEFSC-387, Springfield, VA, USA; 1996.

26. Ilse LM, Hellgren EC. Spatial use and group dynamics of sympatric collared peccaries and feral hogs in southern Texas. Journal of Mammalogy. 1995;76(4):993-1002.

27. Seward NW, VerCauteren KC, Witmer GW, Engeman RM. Feral swine impacts on agriculture and the environment. Sheep & Goat Research Journal. 2004:12.

28. Reidy MM, Campbell TA, Hewitt DG. Evaluation of electric fencing to inhibit feral pig movements. The Journal of Wildlife Management. 2008;72(4):1012-8.

29. Andrzejewski R, Jezierski W. Management of a wild boar population and its effects on commercial land. Acta Theriologica. 1978;23(19):309-39.

30. Meynhardt H. Wildversorgung, Trophäen und Schadensverhütung: Neumann-Neudamm; 1991.

31. Vassant J. Les techniques de prevention des degats de sangliers. Bulletin Mensuel de l'Office National de la Chasse. 1994.

32. Calenge C, Maillard D, Fournier P, Fouque C. Efficiency of spreading maize in the garrigues to reduce wild boar (Sus scrofa) damage to Mediterranean vineyards. European Journal of Wildlife Research. 2004;50(3):112-20.

33. Geisser H, Reyer H-U. Efficacy of hunting, feeding, and fencing to reduce crop damage by wild boars. Journal of Wildlife Management. 2004;68(4):939-46.

34. Groot Bruinderink G, Hazebroek E, Van Der Voot H. Diet and condition of wild boar, Sus scrofu scrofu, without supplementary feeding. Journal of Zoology. 1994;233(4):631-48.

35. Baubet E, Servanty S, Brandt S, Toïgo C, Klein F. Améliorer la connaissance du fonctionnement démographique des populations de sangliers: vers une meilleure gestion de l'espèce Sus scrofa. Rapport scientifique ONCFS. 2004:1-33.

36. Hess SC, Jeffrey JJ, Ball DL, Babich L, Unit HCS. Efficacy of feral pig removals at Hakalau Forest National Wildlife Refuge. Trans West Sect Wildl Soc. 2006;42:53-67.

37. Caley P, Ottley B. The effectiveness of hunting dogs for removing feral pigs (Sus scrofa). Wildlife Research. 1995;22(2):147-54.

38. Saunders G, Bryant H. The evaluation of a feral pig eradication program during a simulated exotic disease outbreak. Wildlife Research. 1988;15(1):73-81.

39. Saunders G. Observations on the effectiveness of shooting feral pigs from helicopters. Wildlife Research. 1993;20(6):771-6.

40. Campbell TA, Long DB. Feral swine damage and damage management in forested ecosystems. Forest Ecology and Management. 2009;257(12):2319-26. doi: 10.1016/j.foreco.2009.03.036.

41. Choquenot D, Kilgour RJ, Lukins BS. An evaluation of feral pig trapping. Wildlife Research. 1993;20(1):15-21.

42. Saunders G, Kay B, Nicol H. Factors affecting bait uptake and trapping success for feral pigs (Sus scrofa) in Kosciusko National Park. Wildlife Research. 1993;20(5):653-65.

43. Sullivan S. Management and Impacts of Wild Hogs (Sus scrofa) in South Carolina: Clemson University, Clemson, South Carolina; 2015.

44. O'Brien PH. An approach to the design of target-specific vertebrate pest control systems: New South Wales Department of Agriculture, Trangie, Australia; 1986.

45. Peters RA. Croonian lecture: lethal synthesis. Proceedings of the Royal Society of London Series B, Biological Sciences. 1952:143-70.

46. Cowled BD, Gifford E, Smith M, Staples L, Lapidge SJ. Efficacy of manufactured PIGOUT® baits for localised control of feral pigs in the semi-arid Queensland rangelands. Wildlife Research. 2006;33(5):427-37.

47. Sherley M. Is sodium fluoroacetate (1080) a humane poison? Animal Welfare-Potters Bar then Wheathampstead. 2007;16(4):449.

48. Bradberry S. Methaemoglobinaemia. Medicine. 2012;40(2):59-60.

49. Wright RO, Lewander WJ, Woolf AD. Methemoglobinemia: etiology, pharmacology, and clinical management. Annals of Emergency Medicine. 1999;34(5):646-56.

50. Shapiro L, Eason C, Bunt C, Hix S, Aylett P, MacMorran D. Efficacy of encapsulated sodium nitrite as a new tool for feral pig management. Journal of Pest Science. 2015:1-7.

51. Snow NP, Halseth JM, Lavelle MJ, Hanson TE, Blass CR, Foster JA, et al. Bait Preference of Free-Ranging Feral Swine for Delivery of a Novel Toxicant. PloS one. 2016;11(1):e0146712.

52. Martin JC. Investigation of sodium nitrite as a control tool for feral pigs: Sul Ross State University, Alpine, Texas; 2015.

53. Lapidge S, Wishart J, Staples L, Fagerstone K, Campbell T, Eisemann J. Development of a feral swine toxic bait (Hog-Gone®) and bait hopper (Hog-Hopper™) in Australia and the USA: Invasive Animals Cooperative Research Center; 2012.

54. Dzięciołowski R. Reproductive characteristics of feral pigs in New Zealand. Acta Theriologica. 1992;37(3):259-70.

55. Comer CE, Mayer JJ. Wild pig reproductive biology. Wild pigs: biology, damage, control techniques, and management (JJ Mayer and IL Brisbin, Jr, editors) SRNL-RP-2009-00869 Savannah River National Laboratory, Aiken, South Carolina. 2009:51-75.

56. Coblentz B, Baber D. Biology and control of feral pigs on Isla Santiago, Galapagos, Ecuador. Journal of Applied Ecology. 1987:403-18.

57. Warren RJ, Ford CR, editors. Diets, nutrition, and reproduction of feral hogs on Cumberland Island, Georgia. Proceedings Annual Conference Southeast Association of Fish and Wildlife Agencies; 1997.

58. Taylor RB, Hellgren EC, Gabor TM, Ilse LM. Reproduction of Feral Pigs in Southern Texas. Journal of Mammalogy. 1998;79(4):1325-31. doi: 10.2307/1383024.

59. Saunders G. The Demography of Feral Pigs (Sus Scrofa) in Kosciusko National Park, New South Wales. Wildlife Research. 1993;20(5):559-69.

60. Sweeney JM, Sweeney JR, Provost EE. Reproductive biology of a feral hog population. The Journal of Wildlife Management. 1979;43(2):555-9.

61. Belden R, Frankenberger W, editors. History and biology of feral swine. Proceedings of the Feral Pig Symposium; 1989.

62. Barrett RH. The feral hog on the dye creek ranch, California: University of California, Division of Agricultural Sciences, Davis, California; 1978.

63. Hampton JO, SPENCER P, Alpers DL, Twigg LE, Woolnough AP, Doust J, et al. Molecular techniques, wildlife management and the importance of genetic population

structure and dispersal: a case study with feral pigs. Journal of Applied Ecology. 2004;41(4):735-43.

64. Mauget R, Campan R, Spitz F, Dardaillon M, Janeau G, Pepin D, editors. Synthèse des connaissances actuelles sur la biologie du sanglier, perspectives de recherche. Symposium international sur le Sanglier Toulouse (France); 1984.

65. Giles J. The ecology of feral pigs in western New South Wales: publisher not identified; 1980.

66. Mellish JM, Sumrall A, Campbell TA, Collier BA, Neill WH, Higginbotham B, et al. Simulating Potential Population Growth of Wild Pig,Sus scrofa, in Texas. Southeastern Naturalist. 2014;13(2):367-76. doi: 10.1656/058.013.0217.

67. Cowan DP, Massei G, editors. Wildlife contraception, individuals and populations: how much fertility control is enough. Proceedings 23rd Vertebrate Pest Conference'(Eds RM Timm and MB Madon) pp; 2008.

68. Burton JL, Westervelt JD, Ditchkoff S. Simulation of Wild Pig Control via Hunting and Contraceptives. DTIC Document, 2013.

69. Almeida FF, Leal MC, França LR. Testis morphometry, duration of spermatogenesis, and spermatogenic efficiency in the wild boar (Sus scrofa scrofa). Biology of Reproduction. 2006;75(5):792-9.

70. ROOIJ DG, RUSSELL LD. All you wanted to know about spermatogonia but were afraid to ask. Journal of Andrology. 2000;21(6):776-98.

71. Hinton B. What does the epididymis do and how does it do it. Journal of Andrology. 1995:18-20.

72. AMANN RP. A critical review of methods for evaluation of spermatogenesis from seminal characteristics. Journal of Andrology. 1981;2(1):37-58.

73. Black J, Erickson B. Oogenesis and ovarian development in the prenatal pig. The Anatomical Record. 1968;161(1):45-55.

74. Ding W, Wang W, Zhou B, Zhang W, Huang P, Shi F, et al. Formation of primordial follicles and immunolocalization of PTEN, PKB and FOXO3A proteins in the ovaries of fetal and neonatal pigs. Journal of Reproduction and Development. 2010;56(1):162-8.

75. Adhikari D, Zheng W, Shen Y, Gorre N, Hämäläinen T, Cooney AJ, et al. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Human Molecular Genetics. 2010;19(3):397-410.

76. Zhang H, Risal S, Gorre N, Busayavalasa K, Li X, Shen Y, et al. Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice. Current Biology. 2014;24(21):2501-8.

77. Morbeck DE, Esbenshade KL, Flowers WL, Britt J. Kinetics of follicle growth in the prepubertal gilt. Biology of Reproduction. 1992;47(3):485-91.

78. Knox R. Recruitment and selection of ovarian follicles for determination of ovulation rate in the pig. Domestic Animal Endocrinology. 2005;29(2):385-97.

79. Schwarz T, Kopyra M, Nowicki J. Physiological mechanisms of ovarian follicular growth in pigs—a review. Acta Veterinaria Hungarica. 2008;56(3):369-78.

80. Bazer FW, Ott TL, Spencer TE. Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology. 1994;41(1):79-94.

81. Bazer FW, Thatcher W. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2α by the uterine endometrium. Prostaglandins. 1977;14(2):397-401.

82. Fagerstone K. Wildlife fertility control: USDA/APHIS/Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado; 2002.

83. Massei G, Cowan D. Fertility control to mitigate human–wildlife conflicts: a review. Wildlife Research. 2014;41(1):1-21.

84. Turner Jr JW, Liu IK, Kirkpatrick JF. Remotely delivered immunocontraception in captive white-tailed deer. The Journal of Wildlife Management. 1992:154-7.

85. Miller L, Rhyan J, Killian G. Evaluation of GnRH contraceptive vaccine using domestic swine as a model for feral hogs: USDA/APHIS/Wildlife Services, National Wildlife Research Center; 2003.

86. Massei G, Cowan DP, Coats J, Bellamy F, Quy R, Pietravalle S, et al. Long-term effects of immunocontraception on wild boar fertility, physiology and behaviour: USDA/APHIS/Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado: 2012;39(5):378-85.

87. Killian G, Miller L, Rhyan J, Doten H. Immunocontraception of Florida feral swine with a single-dose GnRH vaccine. Am J Reprod Immunol. 2006;55(5):378-84. doi: 10.1111/j.1600-0897.2006.00379.x. PubMed PMID: 16635212.

88. Fearneyhough MG. The Texas Oral Rabies Vaccination Project And The Experimental Use Of Raboral V-RG Rabies Vaccine In The South Texas Coyote Rabies Epizootic. 1996.

89. Sanders DL, Xie F, Mauldin RE, Hurley JC, Miller LA, Garcia MR, et al. Efficacy of ERL-4221 as an ovotoxin for feral pigs (Sus scrofa). USDA/APHIS/Wildlife Services, National Wildlife Research Center. 2011;38(2):168-72.

90. Dyer CA, Raymond-Whish S, Schmuki S, Fisher T, Pyzyna B, Bennett A, et al. Accelerated follicle depletion in vitro and in vivo in Sprague-Dawley rats using the combination of 4-vinylcyclohexene diepoxide and triptolide. Journal of Zoo and Wildlife Medicine. 2013;44(4s):S9-S17.

91. Tao X, Younger J, Fan FZ, Wang B, Lipsky PE. Benefit of an extract of Tripterygium Wilfordii Hook F in patients with rheumatoid arthritis: A double- blind, placebo- controlled study. Arthritis & Rheumatism. 2002;46(7):1735-43.

92. Chen BJ. Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb Tripterygium wilfordii Hook F. Leukemia & Lymphoma. 2001;42(3):253-65.

93. Yang S, Chen J, Guo Z, Xu X-M, Wang L, Pei X-F, et al. Triptolide inhibits the growth and metastasis of solid tumors1. Molecular Cancer Therapeutics. 2003;2(1):65-72.

94. Mayer LP, Devine PJ, Dyer CA, Hoyer PB. The follicle-deplete mouse ovary produces androgen. Biology of Reproduction. 2004;71(1):130-8.

95. Mayer LP, Dyer CA, Eastgard RL, Hoyer PB, Banka CL. Atherosclerotic lesion development in a novel ovary-intact mouse model of perimenopause. Arteriosclerosis, Thrombosis, and Vascular biology. 2005;25(9):1910-6.

96. Thomas FH, Vanderhyden BC. Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. Reproductive Biology and Endocrinology. 2006;4(1):1.

97. Mark-Kappeler CJ, Sen N, Lukefahr A, McKee L, Sipes IG, Konhilas J, et al. Inhibition of ovarian KIT phosphorylation by the ovotoxicant 4-vinylcyclohexene diepoxide in rats. Biology of Reproduction. 2011;85(4):755-62.

98. Xu C-k, Zhao Y-h. Apoptosis of rats ovarian follicle cells induced by triptolide in vivo. African Journal of Pharmacy and Pharmacology. 2010;4(6):422-30.

99. Li W, Liu Y, He Y-Q, Zhang J-W, Gao Y, Ge G-B, et al. Characterization of triptolide hydroxylation by cytochrome P450 in human and rat liver microsomes. Xenobiotica. 2008;38(12):1551-65.

100. Hayes JD, McLELLAN LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radical Research. 1999;31(4):273-300.

101. Zeng Y, Sun H, Li Y, Shao M, Han P, Yu X, et al. Exposure to triptolide affects follicle development in NIH mice Role of endoplasmic reticulum stress in granulosa cell apoptosis. Human & Experimental Toxicology. 2016:0960327116638725.

102. Yu Z, Luo H, Fu W, Mattson MP. The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Experimental Neurology. 1999;155(2):302-14.

103. Huynh PN, Hikim AS, Wang C, Stefonovic K, Lue Y, Leung A, et al. Long-term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. Journal of Andrology. 2000;21(5):689-99.

104. Hikim A, Lue Y, Wang C, Reutrakul V, Sangsuwan R, Swerdloff RS. Posttesticular antifertility action of triptolide in the male rat: evidence for severe impairment of cauda epididymal sperm ultrastructure. J Androl. 2000;21(3):431-7.

105. Xue M, Zhao Y, Li X-j, Jiang Z-z, Zhang L, Liu S-h, et al. Comparison of toxicokinetic and tissue distribution of triptolide-loaded solid lipid nanoparticles vs free triptolide in rats. European Journal of Pharmaceutical Sciences. 2012;47(4):713-7.

106. Hu J, Yu Q, Zhao F, Ji J, Jiang Z, Chen X, et al. Protection of Quercetin against Triptolide-induced apoptosis by suppressing oxidative stress in rat Leydig cells. Chemico-Biological Interactions. 2015;240:38-46. doi: http://dx.doi.org/10.1016/j.cbi.2015.08.004.

107. Ma B, Qi H, Li J, Xu H, Chi B, Zhu J, et al. Triptolide disrupts fatty acids and peroxisome proliferator-activated receptor (PPAR) levels in male mice testes followed by testicular injury: a GC–MS based metabolomics study. Toxicology. 2015;336:84-95.

108. Hooser SB, Demerell DG, Douds DA, Hoyer P, Sipes IG. Testicular germ cell toxicity caused by vinylcyclohexene diepoxide in mice. Reproductive Toxicology. 1995;9(4):359-67.

109. Lue Y, Sinha Hikim A, Wang C, Leung A, Baravarian S, Reutrakul V, et al. Triptolide: a potential male contraceptive. Journal of Andrology. 1998;19:479-86.

110. Mayer LP, Dyer CA. Reducing the reproductive capacity of mammals. Google Patents; 2013.

111. Campbell TA, Long DB, Massei G. Efficacy of the Boar-Operated-System to deliver baits to feral swine: USDA/APHIS/Wildlife Services, National Wildlife Research Center; Prev Vet Med. 2011;98(4):243-9. doi: 10.1016/j.prevetmed.2010.11.018. PubMed PMID: 21176854.

112. Kavanaugh D, Linhart S. A modified bait for oral delivery of biological agents to raccoons and feral swine. Journal of Wildlife Diseases. 2000;36(1):86-91.

113. Campbell TA, Long DB. Mammalian visitation to candidate feral swine attractants. The Journal of Wildlife Management. 2008;72(1):305-9.

114. Martinez EA, Vazquez JM, Matas C, Gadea J, Alonso M, Roca J. Oocyte penetration by fresh or stored diluted boar spermatozoa before and after in vitro capacitation treatments. Biology of Reproduction. 1996;55(1):134-40.

115. Griffin J, Emery BR, Huang I, Peterson CM, Carrell DT. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). Journal of Experimental & Clinical Assisted Reproduction. 2006;3(1):2.

116. Campbell TA, Long DB. Species-specific visitation and removal of baits for delivery of pharmaceuticals to feral swine. Journal of Wildlife Diseases. 2007;43(3):485-91.

117. Burd AM. In vivo and in vitro studies of 4-vinylcyclohexene diepoxide in wild-caught female brushtail possums (Trichosurus vulpecula) and Norway rats (Rattus norvegicus) and its potential as a fertility control agent: Lincoln University; 2014.

118. Gadea J, Matás C, Lucas X. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay: University of Murcia; Animal Reproduction Science. 1998;54(2):95-108.

119. Youngquist RS, Threlfall WR. Current Therapy in Large Animal Theriogenology: Elsevier Health Sciences; 2006.

120. Yang F, Ren L, Zhuo L, Ananda S, Liu L. Involvement of oxidative stress in the mechanism of triptolide-induced acute nephrotoxicity in rats. Experimental and Toxicologic Pathology. 2012;64(7):905-11.

121. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biology of Reproduction. 1989;41(1):183-97.

122. Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. Molecular and Cellular Endocrinology. 2004;216(1–2):31-9. doi: http://dx.doi.org/10.1016/j.mce.2003.10.069.

123. He B, Guo H, Gong Y, Zhao R. Lipopolysaccharide-induced mitochondrial dysfunction in boar sperm is mediated by activation of oxidative phosphorylation. Nanjing Agricultural University, Nanjing, China; Theriogenology. 2016.

124. Brito LF. Evaluation of stallion sperm morphology. Clinical Techniques in Equine Practice: Univeristy of Pennsylvania; 2007;6(4):249-64.

125. Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, et al. Mechanism of extracellular ubiquitination in the mammalian epididymis. Journal of Cellular Physiology. 2008;215(3):684-96.

126. Ni B, Jiang Z, Huang X, Xu F, Zhang R, Zhang Z, et al. Male reproductive toxicity and toxicokinetics of triptolide in rats. Arzneimittelforschung. 2008;58(12):673-80.

127. Huang Z, Que H, Peng H, Lin S, Guo S, Qian L. [Reproductive toxicity of triptolide and its mechanism in male rats]. Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China Journal of Chinese Materia Medica. 2015;40(23):4655-9.

128. Foresta C, Flohé L, Garolla A, Roveri A, Ursini F, Maiorino M. Male fertility is linked to the selenoprotein phospholipid hydroperoxide glutathione peroxidase. Biology of Reproduction. 2002;67(3):967-71.

129. Olson GE, Winfrey VP, Hill KE, Burk RF. Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium-deficient rats. Reproduction. 2004;127(3):335-42.

130. Du F, Liu Z, Li X, Xing J. Metabolic pathways leading to detoxification of triptolide, a major active component of the herbal medicine Tripterygium wilfordii. Journal of Applied Toxicology. 2014;34(8):878-84.

131. Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, et al. Dual function of the selenoprotein PHGPx during sperm maturation. Science. 1999;285(5432):1393-6.

132. Flohe L. Selenium in mammalian spermiogenesis. Biological Chemistry. 2007;388(10):987-95.

133. Schneider M, Förster H, Boersma A, Seiler A, Wehnes H, Sinowatz F, et al. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. The FASEB journal. 2009;23(9):3233-42.

134. King R, Williams I. The influence of ovulation rate on subsequent litter size in sows. Theriogenology. 1984;21(4):677-80.

135. Roosa KA, Zysling DA, Place NJ. An assessment of anti-Müllerian hormone in predicting mating outcomes in female hamsters that have undergone natural and chemically-accelerated reproductive aging. General and Comparative Endocrinology. 2015;214:56-61.

136. Sahambi SK, Visser JA, Themmen AP, Mayer LP, Devine PJ. Correlation of serum anti-Mullerian hormone with accelerated follicle loss following 4-vinylcyclohexene diepoxide-induced follicle loss in mice. Reprod Toxicol. 2008;26(2):116-22. doi: 10.1016/j.reprotox.2008.07.005. PubMed PMID: 18706995.

137. Monniaux D, Clément F, Dalbiès-Tran R, Estienne A, Fabre S, Mansanet C, et al. The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link? Biology of Reproduction. 2014;90(4):85.

138. Almeida F, Teerds K, Auler P, Soede N. Anti-Mullerian hormone: expression and possible roles in the porcine ovary. Proceedings of the 17th International Congress on Animal Reproduction, Vancouver, Canada: Reproduction in Domestic Animals; 2012.